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AMYLOIDOSIS IN MICE PRODUCED BY TRANSPLANTATION OF SPLEEN CELLS FROM CASEIN-TREATED MICE

By

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Received 8 iii 66

Experimental amyloidosis may be produced by repeated injections of antigenic material Injections of sodium caseinate in mice, as described by *Kuczynski* (1922), has proved to be a very effective method In our laboratory a 5 per cent solution of casein in 0 25 per cent Na OH is injected subcutaneously in mice five or six times weekly In the course of 14 to 35 injections spleen amyloidosis regularly sets in (*Christensen* 1963)

Amyloidosis induced by casein treatment can be accelerated or enhanced by treatment with cortisone or ACTH (*Teilum* 1952), by treatment with nitrogen mustard (*Teilum* 1954), and by x-irradiation (*Christensen & Hjort* 1959, 1960) When nitrogen mustard is administered to C3H mice pretreated with casein, a diffuse and massive spleen amyloidosis rapidly ensues In the original experiment (*Teilum* 1954) the treatment was 28 hypodermic casein injections given in the course of 30 days, which were followed by three hypodermic injections of 0 05 mg of nitrogen mustard given in the course of eight days The accelerating effect of the nitrogen mustard treatment was shown by histological comparison of the spleen after the treatment, with spleen biopsies taken before the treatment

Recently, 'the two-phase cellular theory of local secretion' for the pathogenesis of amyloidosis, has been introduced (*Teilum* 1964) The essence of the theory is that amyloid is produced *in situ* by cellular secretion The appearance of amyloid in a tissue—the amyloid phase—is always preceded by pyroninophilic cell proliferations in the same tissue—the pyroninophilic phase

Experiments using cortisone or nitrogen mustard as accelerators of casein induced amyloidosis in mice, are good models of this bi phasic development During the treatment with casein an increasing prolifer-

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ation of cells showing cytoplasmatic pyroninophilia is obvious and most readily studied in the spleen. When the treatment with cortisone or nitrogen mustard begins, the number of pyroninophilic cells rapidly decreases and amyloid is produced. In such experiments the transitory occurrence of periodic acid-Schiff positive reticular cells has been observed on the border of and inside the new-formed amyloid, and they are believed to be directly involved in the production of amyloid (*Teilum* 1956).

Recently new formation of amyloid has been observed in tissue cultures of human thyroid carcinoma, and evidence presented that the amyloid is produced by secretion from PAS-positive tumour cells (*Albores Saavedra et al.* 1964). *Cohen, Gross & Shirahama* (1964, 1965) have observed PAS positive cells in tissue cultures of amyloid laden rabbit spleens maintained for up to forty-five days.

Various experimental techniques employed in transplantation biology may lead to amyloidosis. A transplantable reticulosarcoma in mice, accompanied by generalized amyloidosis, has been reported by *Rask-Nielsen, Christensen & Clausen* (1960). Amyloidosis in parabiotic mice has been described by *Hall, Hall & Cross* (1959) and by *Williams* (1954), and in Syrian hamsters by *Walford & Hildeman* (1964). Amyloidosis has been observed in radiation chimeras, too (*Bradbury & Micklem* 1965). In these experiments the occurrence of amyloidosis has been interpreted as the result of a prolonged stimulation of the immune mechanisms of the amyloidotic animals.

In order to study the role of cells in the pathogenesis of amyloidosis we have isolated spleen cells in pyroninophilic phase and transferred them to normal syngeneic recipients which are then treated with an amyloidosis accelerating substance. We believed that the transferred cells might colonize the new host and—under the influence of the amyloidosis accelerating treatment—produce amyloid in the host.

In the present experiments spleen cells from casein treated C3H mice are transferred to normal syngeneic recipients, which then receive treatment with nitrogen mustard. The lesions thus produced are studied histologically, and the pathogenesis of experimental amyloidosis is discussed in the light of the present and earlier investigations.

MATERIAL AND METHODS

Young C3H mice five to ten weeks old weighing 15-25 grammes and of both sexes were used throughout the experiments. The mice are of an inbred strain with a low incidence of spontaneous amyloidosis.

Donors: the mice were fed on oatmeal and water *ad libitum*. Groups of donors, from seven to twenty mice at a time, received hypodermic injections of 5 per cent casein in 0.25 per cent NaOH prepared according to the method adopted by *Christensen* (1963). A daily dose of 0.5 ml was given six days a week in different parts of the back. After eleven to twenty casein injections the animals were killed by cervical dislocation on the day of the last injection. The spleens were taken out immediately, freed from adjacent fat and pancreatic tissue, and placed in Ringer solution.

Spleen cell suspension: the donor spleens were found to be enlarged to about twice

the normal size From each spleen a piece was taken for microscopy, while the main part was cut open All the spleens were pooled in a loose fitting glass homogenizer and Ringer solution was added to about ten times the volume of the spleens The piston was moved up and down gently crushing of the cells being carefully avoided In this way the spleen cells readily separate from the pulp The suspension was filtered through a small metal sieve (1600 holes per cm^2) The filtrate was centrifuged for five minutes at 1500 revolutions per minute resuspended in Ringer solution then centrifuged and resuspended twice more Counting of the suspension in counting chamber revealed the number of nucleated cells per ml usually between 200×10^6 and 300×10^6 Ringer solution was added so that the final suspension contained 200×10^6 cells per ml The viability of the cell suspension was controlled in each experiment with isotonic trypan blue solution and found to range from 80 to 90 per cent

Recipients All mice were healthy and in the natural state at the time of the cell transfer They were given 200×10^6 or—in some instances—100×10^6 cells by injection into the tail vein Treatment with nitrogen mustard (Erasol IDO®) was started on the day of the cell transfer it was given as hypodermic injections of 0 05 mg either every or every second day for up to seven days whereupon the animals were killed by cervical dislocation

Histological methods At autopsy spleen liver kidney adrenal and lung were taken out fixed in ten per cent neutral formalin and embedded in paraffin From animals selected at random thymus mesenteric lymph node cardiac muscle pancreas and intestine were taken as well Sections were stained with haematoxylin eosin Van Gieson Hansen stain pyronin methyl green periodic acid Schiff technique methyl violet Congo red and thioflavine T For the technique specificity and applications of the thioflavine T staining method the reader is referred to *Vassar & Culling* (1959), *Janigan* (1965) and *Rogers* (1965)

Control experiments Several control experiments were carried out in order to study the following matters 1) the morphogenesis of the spleen amyloidosis in recipients of spleen cells from casein treated donors 2) the effect of nitrogen mustard on mice receiving normal spleen cells 3) the effect of nitrogen mustard on mice receiving no cells 4) the outcome produced by the transfer of cells from casein treated mice to mice which are left untreated and finally 5) the amyloidosis enhancing effect of nitrogen mustard on casein induced amyloidosis The casein treatment the preparation of the the cell suspension the method of the cell transfer and the histolog cal methods employed in the control experiments are the same as described under the main experiment The exact treatment of the animals in these groups was as follows

1) 51 mice each received 100×10^6 spleen cells from donor mice treated with 17 casein injections Treatment with nitrogen mustard was started on the day of the cell transfer and given as injections of 0 05 mg every second day The recipient mice were killed and autopsied in groups of eight 0 1 2 3 4 and 5 days after the cell transfer

2) 11 mice each received a transfusion of 200×10^6 spleen cells from normal donor mice Nitrogen mustard was administered as described above Five days after the cell transfer the animals were killed and autopsied

3) 9 normal mice received treatment with nitrogen mustard like the recipients in group 1 After five days treatment they were killed and autopsied

4) 6 normal mice received a transfusion of 200×10^6 spleen cells from donor mice treated with 14 casein injections The recipients were left to themselves without treatment for five days killed and autopsied

5) 5 separate groups 26 mice in all, were treated with 11 14 17 20 and 30 casein [illegible] d was started on the day [illegible] erent periods of time and [illegible] s after the last nitrogen

Estimation of Histological Lesions

Spleen lung liver, and kidney were available for study from all animals Screening for fluorescence in ultraviolet light in thioflavine T stained sections was found to be a very suitable method for the

detection of amyloid especially when this was sparse. Only bright yellow fluorescent material was taken into account as amyloid. Good agreement was found between the thioflavine T stain and the other staining methods employed. Amyloid was only diagnosed as such when it could be detected in all the differently stained sections from the same block.

A rough quantitation of the spleen amyloidosis has been carried out in accordance with the method employed by *Christensen & Hjort* (1959). This method rests upon an estimation of the breadth of the amyloid rings in the perifollicular zone round the splenic follicles. Grades 2 to 6 express increasing degrees of amyloidosis. Grade 2 denotes the narrowest rings of amyloid, grade 6 a maximum of amyloidosis accompanied by complete obliteration of the normal spleen structure. The quantitation was carried out on the PAS stained sections.

RESULTS

Transfer of Spleen Cells from Casein Treated Mice to Mice Subsequently Treated with Nitrogen Mustard (Main Experiment)

TABLE 1

Incidence of Spleen Amyloidosis in Nitrogen Mustard Treated Recipient Mice after Inoculation of Spleen Cells from Casein Treated Mice (Main Experiment)

Number of casein injections given to donor mice	Number of donor spleens with amyloidosis	Number of injected spleen cells per recipient × 10^6	Treatment with nitrogen mustard (days)	Number of recipient mice with spleen amyloidosis
11	0/11	200	5	0/5
14	0/7	120	4	0/6
14	2/14	200	5	13/14
17	1/5	100	5	7/8
20	4/14	100	7	5/6
20	4/14	200	4	4/4
20	4/14	200	7	6/7

The experimental conditions and results of the main experiment are tabulated in Table 1. It will be seen that in most of the groups the recipient mice have developed spleen amyloidosis with great regularity. Thus, omitting the group which received cells from mice treated with only 11 casein injections, 35 out of 45 recipients developed amyloidosis.

Morphologically the spleen amyloid is found as irregular rings with shaggy borders in the perifollicular zone round the splenic follicles (Figs. 1 and 2). The amyloid substance contains many bloodfilled spaces giving a vacuolated appearance, and a netlike pattern is visible which appears to be remnants of the splenic reticulum. The amyloid

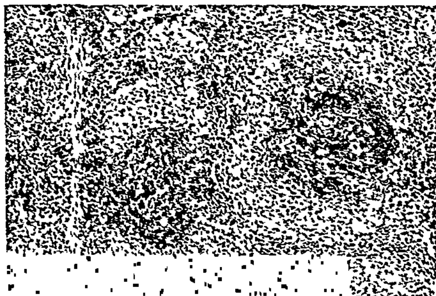


Fig 1

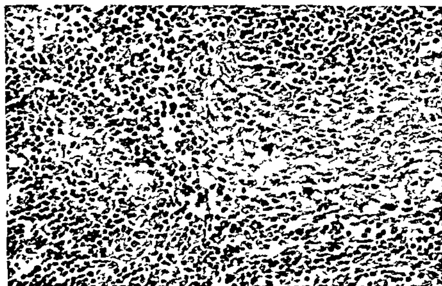


Fig 2

Spleen amyloidosis in mouse treated with nitrogen mustard for seven days after the transfer of 100×10^6 spleen cells from donors treated with 20 casein injections Note the cellularity of the amyloid material Periodic acid Schiff stain ($\times$ 350)



Figs 3-8

substance contains numerous reticular cell nuclei, some of which are distorted and pycnotic The cellularity of the amyloid is notable as compared with the scarcity of cells in the amyloid found in the control group with ordinary nitrogen mustard accelerated amyloidosis (Fig 17) *The red and white pulp is shrunken and depleted of cells,* and the number of pyroninophilic cells is small In most of the spleens many haemosiderin laden macrophages are seen in the red pulp In the periodic acid Schiff stained preparations are found great numbers of PAS-positive reticular cells with vacuolated and granular cytoplasm (Figs 4 5) Most of these cells are in the red pulp close to the borders of the amyloid rings, and in some instances there is direct transition from PAS positive cells to amyloid (Fig 6) Reticular cells with PAS-positive cytoplasm fading into the amyloid may even be surrounded by the amyloid substance itself (Fig 7) As for quantity, in the spleens where it is found, the amyloidosis is of degree 2 to 3 In mice treated with nitrogen mustard after a period of casein treatment the average degree of spleen amyloidosis is considerably higher, between 4 and 5

Histochemically the amyloid is weakly Congo-positive, moderately PAS-positive The PAS positivity is strongest in the periphery of the amyloid rings Staining with methyl violet reveals no metachromasia *The amyloid shows bright yellow fluorescence in ultraviolet light* in thioflavine T-stained preparations (Fig 8) and weak green birefringence in Congo stained preparations, when studied through crossed polars The histochemical characteristics of the recipients amyloid are the same as those found in spleens from the control group with nitrogen mustard accelerated casein amyloidosis, except that it is more PAS positive

In the recipient mice that do not develop amyloidosis great numbers of PAS-positive reticular cells are found in the perifollicular zone of the white pulp (Fig 4) These cells are similar to the PAS cells found adjacent to the amyloid deposits in most recipients Such cells however, are also found in controls which have received normal cells prior to the nitrogen mustard treatment, though in smaller numbers

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Figs 3–8

Fig 3 Donor spleen on the day of cell transfer Pronounced cytoplasmatic pyroninophilia of reticular cells in the splenic red pulp (Pyronin methyl green stain × 380)

Figs 4–8 Spleens from recipient mice having received spleen cells from casein treated donors and nitrogen mustard treatment *Fig 4* shows PAS positive cells perifollicularly in a recipient which has not yet developed amyloidosis (PAS stain × 238) *Fig 5* shows PAS positive cells on the border

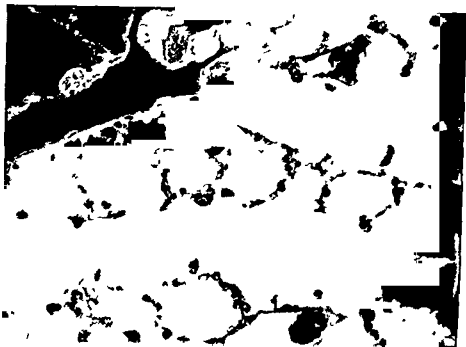


Fig 9

Amyloid emboli in lung vessels Mouse treated with nitrogen mustard for six days after the cell transfer Some of the donor mice contributing to this cell suspension had developed amyloidosis Thioflavine T stain fluorescence in ultraviolet light (× 350)

In control mice treated with nitrogen mustard alone, these cells are few

The mice, 4 in all which died less than 96 hours after the cell transfer, have been omitted from the groups None of these developed spleen amyloidosis

In the liver and kidneys of the recipients amyloid was never encountered, but in the lungs from most of the recipients listed in Table 1 many small lumps of amyloid were found in the vessels The amyloid in the lungs is found intravasally in the small vessels and the alveolar capillaries as round or oval bodies lying free in or, more often, expanding the vessels (Fig 9) Very few cell nuclei are found in these emboli, in some none at all Amyloid is not found in the vessel wall or in the lung parenchyme The histochemical reactions of these amyloid emboli were found to be precisely the same as those of the spleen amyloid in the corresponding donor mice A comparison between amyloid emboli in the lungs of mice dying immediately after the cell transfer and amyloid emboli found in the lungs of mice killed after five days nitrogen mustard treatment, does not reveal any difference with regard to quantity or histological and histochemical properties

The occurrence of amyloid emboli in the lungs of the recipient mice and the mortality immediately after the cell transfer are related to the

state of the donor spleens used When many of the donor spleens contain amyloid the mortality following immediately upon the cell transfer is high and many amyloid emboli are found in the recipients lungs and *vice versa* Amyloid emboli have not been found in the systemic circulation of any of the recipient mice

The condition with regard to amyloidosis of the donor spleens employed in the transfer experiments is shown in Table 1 After 11 casein injections none of the donors have developed spleen amyloidosis while after 17 or 20 casein injections several of them have

Apart from the spleen amyloidosis of degrees 2 or 3 seen in some donor spleens their outstanding histological feature is a heavy proliferation of pyroninophilic cells which crowd the splenic red pulp (Fig 3) The pyroninophilic cells are mostly reticular cells with pale vesicular nuclei each containing a pyroninophilic nucleus The reticular cells are interspersed with small numbers of mature plasma cells and some Russell bodies as well Very few PAS positive cells are found With regard to the degree of pyroninophilic cell proliferation and the frequency of PAS-positive cells no difference can be detected between the spleens from donors treated with 11, 14 17, or 20 casein injections

The relation between donor spleen amyloidosis and the occurrence of amyloidosis in the corresponding group of recipients is clearly shown in Table 1 When some of the donor mice had developed amyloidosis most of the corresponding recipients did develop amyloidosis too On the other hand if none of the donor mice had spleen amyloidosis none of the corresponding recipients developed it either

TABLE 2

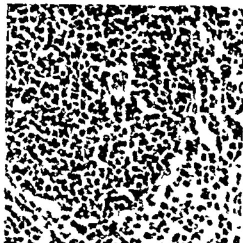
Incidence of Spleen Amyloidosis in Recipient Mice Killed at Various Intervals after Transfer of Spleen Cells from Casein Treated Mice

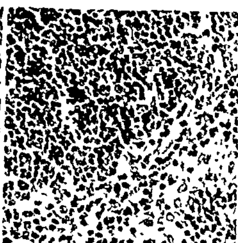
Number of injected spleen cells per recipient × 10^6	Treatment with nitrogen mustard (days)	Number of recipient mice with spleen amyloidosis
100	0*	0 8
100	1	0 8
100	2	6/8
100	3	8 8
100	4	9/9
100	5	10 10

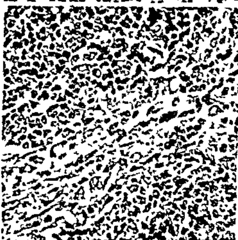
* Died spontaneously within minutes after the cell transfer

Transfer of Spleen Cells from Casein Treated Mice to Mice Killed at Varying Intervals during Treatment with Nitrogen Mustard

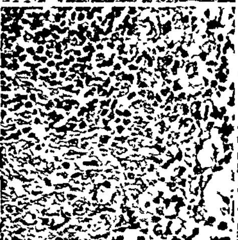
In order to study the morphogenesis of recipient spleen amyloidosis described above the following experiment was designed 51 mice each

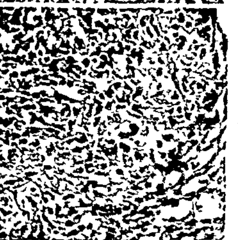
10 

 11

12 

 13

14 

 15

Figs 10-15

received 100×10^6 spleen cells from donor mice treated with 17 casein injections Treatment with nitrogen mustard was started on the day of the cell transfer, and the recipient mice were killed in groups of eight 0, 1, 2, 3, 4, and 5 days after the cell transfer. The result is shown in Table 2 It will be seen that the recipient spleen amyloidosis does not occur until 48 hours after the cell transfer At that time thin, incomplete, strongly PAS positive rings giving the usual histochemical reactions of amyloid may be seen in the perifollicular zone of the splenic white pulp Simultaneously large numbers of PAS-positive reticular cells appear in the perifollicular zone During the following three days the amyloid rings grows broader and eventually all the spleens show amyloidosis of degree 2 or 3 (Figs 10 to 15).

As in other experiments where some of the donor spleens are amyloidotic, 9 out of 18 in this experiment, amyloid emboli are found in the lung vessels of the recipients Amyloid was not found in the liver or kidneys of any of the recipients

Transfer of Spleen Cells from Normal Mice to Mice Subsequently Treated with Nitrogen Mustard

11 mice each received 200×10^6 spleen cells from normal donor mice After five days' treatment with nitrogen mustard amyloid was found in one recipient spleen, but only a single small streak of amyloid measuring approximately 200×60 microns was found in the sections from this mouse It was, however, amyloid of the same histochemical and histological character as the amyloid found in the main experiment In all the spleens from this group moderate numbers of PAS-positive reticular cells were observed in the splenic red pulp

The Effect on Mice of Nitrogen Mustard Treatment Alone

9 normal mice were treated with nitrogen mustard for five days None of these developed amyloidosis The spleens were shrunken and cell depleted, most pronouncedly in the splenic red pulp Very few PAS-positive cells were seen in the splenic pulp

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Figs 10 15

Spleens from mice each inoculated with 100×10^6 spleen cells from donors treated with 17 casein casein injections The recipient mice were subsequently treated with nitrogen mustard and killed at different intervals after the cell transfer *Fig 10* From mouse killed 5 min after the cell transfer *Fig 11* 24 hours after the cell transfer *Fig 12* 48 hours after the cell transfer PAS positive reticular cells and small PAS positive deposits in the perifollicular zone *Fig 13* 72 hours after the cell transfer Confluent PAS positive deposits fully recognizable as amyloid *Figs 14* and *15* 96 hours and 120 hours after the cell transfer respectively Condensation and increasing breadth of amyloid rings in the perifollicular zone (Periodic acid Schiff technique $\times$ 350)

Transfer of Spleen Cells from Casein-Treated Mice to Mice not Further Treated

6 normal mice each received 200 × 10⁶ spleen cells from a donor group of 10 mice treated with 14 casein injections One of the donor mice had spleen amyloidosis

Spleen, liver, and kidneys of all the recipients were free from amyloidosis after five days The splenic pulp appeared normal, and only small numbers of PAS-positive cells were found However, in the lungs of all the recipients small amyloid emboli were found just as in the main experiment

TABLE 3

The Incidence Localization and Degree of Amyloidosis in Mice Treated with Casein Followed by Nitrogen Mustard

Number of mice	Number of casein injections	Number of nitrogen mustard injections /days	Total dose of nitrogen mustard (mg)	Number of mice with amyloidosis in spleen	liver	kidney	Average degree of spleen amyloidosis
3	11	2/3	0 1	1	0	0	3
14	14	3 5	0 15	4	0	0	3
3	17	3/5	0 15	3	3	2	4 5
2	20	4/5	0 20	2	2	0	4 5
4	20	3/6	0 15	4	4	3	4 5

Mice Treated with Casein and Subsequently with Nitrogen Mustard

The results of nitrogen mustard treatment of mice pretreated with casein for different periods of time are shown in Table 3 All animals pretreated with 17 or 20 casein injections developed a massive spleen and liver amyloidosis while animals pretreated with only 14 casein injections did not, except for four out of fourteen, do so These groups however, are not directly comparable, as the duration of the nitrogen mustard treatment was not the same Renal amyloidosis in the form of small glomerular lesions occurred in the groups treated with casein for the longest periods of time

The heavy spleen amyloidosis in some of the groups in Table 3 shows as broad, well circumscribed rings in the periphery of the splenic follicles (Figs 16 and 17) The amyloid has a fibrillar appearance and contains some reticular cell nuclei Many blood filled spaces are contained within the amyloid material Some, not many, PAS-positive reticular cells are found close to the peripheral borders of the amyloid deposits Histochemically the amyloid shows weak reaction with Congo red and the PAS stain It is not metachromatic with methyl violet but gives a bright yellow fluorescence in ultraviolet light with thioflavine T



Fig 16

Spleen amyloidosis degree 4 Mouse treated with nitrogen mustard for six days after 20 casein injections Note the well defined amyloid rings Haematoxylin eosin (× 100)

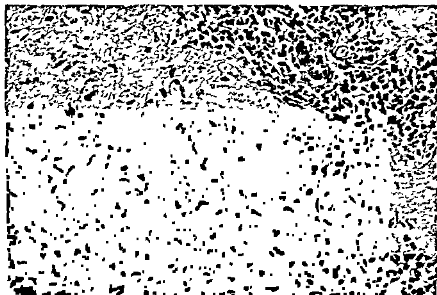


Fig 17

Spleen amyloidosis in mouse treated with nitrogen mustard for four days after 20 casein injections Note the comparatively cell poor amyloid in contrast to the *amyloid in the recipients (Fig 2) Periodic acid Schiff stain (× 350)*

DISCUSSION

Our results show that intravenous inoculation of spleen cells from casein-treated mice to syngeneic mice which are then treated with nitrogen mustard induces amyloidosis in the recipients Under similar conditions transfer of cells from normal mice does not induce amyloidosis in the nitrogen mustard-treated recipients Neither does nitrogen mustard treatment alone

A relevant control experiment would be the transfer of killed spleen cells from casein-treated mice to nitrogen mustard-treated recipients We have tried to kill such donor cells by repeated freezing and thawing and by gentle heating, the result having in each instance been controlled by the trypan blue test These methods have not been successful in our hands, as the procedures employed in order to kill the cells have brought about coagulation of the cell suspension, thus rendering the material uninjectable

Before any conclusions can be reached regarding the mode of amyloid formation in these experiments two problems have to be considered First which components other than spleen cells and Ringer solution make part of the inoculum, and is it possible that such components may reach the spleen of the recipient and contribute to the splenic lesions? Secondly do the inoculated cells colonize the recipient's spleen in such numbers as could be responsible for the spleen amyloidosis in the recipients?

With regard to the first question it may be recalled that the method employed in the preparation of the cell suspension excludes the possibility of a transfer of any humoral factor, as the cell suspension is centrifuged with subsequent resuspension in fresh Ringer solution three times before use The inoculum, however, may contain small lumps of amyloid formed in the donor spleens The larger ones are arrested in the lungs—this is obvious from the lesions found in the recipient's lungs It cannot be excluded, however, that small particles of preformed amyloid may pass the lungs and reach the spleen of the recipient The facts that amyloid is never found in the recipient's spleen until 48 hours after the cell transfer and that recipients not treated with nitrogen mustard never develop amyloidosis, argue against such a mechanism playing any significant part in the establishment of the splenic lesions It should be stressed that amyloid particles were never found in the systemic vessels or in the Kupffer cells of the liver in the recipients

In recent years a number of investigations have been carried out which are closely related to our second problem the fate of intravenously injected reticulo-endothelial cells The use of autoradiography in tracing cells labelled with tritiated or P^{32}-incorporated compounds has been applied to guinea pig spleen cells (*Turk* 1962), rat thoracic duct lymphocytes (*Gowans* 1962, *Gowans, Mc Gregor & Cowen*

1963), thymus cells (*Diderholm* 1961, *Diderholm & Fichtelius* 1959 a), and lymph node cells (*Diderholm & Fichtelius* 1959 b) Among the quoted investigators there is agreement that a considerable part of the injected cells become concentrated in and colonize the spleen *Diderholm* (1961), working with transfer of labelled guinea pig thymus cells finds a higher concentration of the injected cells in the recipient's spleen than in any other organ Next to the spleen in order of concentration of injected cells comes the liver, while only small numbers were found in the bone marrow, lymph nodes, and lungs In transfer experiments *Diderholm & Fichtelius* (1959 a and b) found the highest concentration of labelled thymus and lymph node cells in the splenic white pulp 48 hours after the cell transfer In other experiments *Diderholm* (1961) traces labelled thymus cells to the splenic red pulp eight hours after the transfer, adding that sometimes the cells were concentrated in regions near the white pulp While there is general agreement that inoculated lymphoid cells become concentrated in the spleen of the recipient, varying views have been put forward regarding the exact regions of the spleen which are colonized by the cells *Diderholm* (1961) favours the opinion, cited above that most of the cells settle in the splenic red pulp with some tendency to accumulation in regions near the white pulp, while *Gowans* (1962) traces by far most of the injected cells to the white pulp where they accumulate about the central arteriole of the follicle *Osogoe & Hitachi* (1950) *Osogoe* (1950), and *Karasawa & Osogoe* (1954), working with large numbers of unlabelled rabbit lymph node and thymus cells conclude that the inoculated cells colonize the perifollicular zone of the splenic white pulp and the periportal spaces of the liver

Thus, with regard to our own experiments it can be concluded that no humoral factor or any significant amount of preformed donor amyloid reaches the recipient s spleen and that the injected cells colonize first and foremost the spleen of the recipient The cellularity of the amyloid rings in the recipients' spleens compared with the scarcity of cells in the spleen amyloid in the casein nitrogen mustard treated controls, provides further evidence that the transferred cells colonize the recipient's spleen in the sites of amyloid production The spleen amyloid in our recipients is found as rings in the perifollicular zone of the splenic white pulp, the same pattern as that presented by spleen amyloid in the usual forms of experimental amyloidosis In other respects it differs from this pattern it is more cellular, the rings are thin and irregular, and it appears more vacuolated These morphological dissimilarities suggest that the transferred cells, having colonized the recipient s spleen, are involved locally in the production of the amyloid The tentative conclusion is that the transferred cells colonize the spleen and produce the amyloid locally

Cells which may be concerned in a local production of amyloid are the PAS positive reticular cells on the border of and within the amyloid

material, as described by *Teilum* (1956) Such cells are also frequently found in our recipients Without transfusion of labelled donor cells, however, it cannot be settled whether these PAS-positive cells, found in our recipients, really are transferred donor cells

In the ordinary amyloidosis acceleration experiment the casein immunization and the nitrogen mustard treatment are given to the same animal In our transfer experiment the immunization and the nitrogen mustard treatment are dissociated, and only the transferred spleen cells have been under the influence of both This may be looked upon as an illustration of the biphasic development of amyloid referred to in the introduction

The absence of amyloid development in liver and kidney is in accordance with the assumption that amyloid is produced locally by the transferred cells. The amount of transferred cells corresponds to one or two normal spleens The cells, however, are distributed into the tissues of the entire mouse and are concentrated in appreciable numbers in the spleen only The lumps of amyloid found in the lungs of recipients in most of the groups are amyloid emboli preformed in the donor mice When the donor spleens of which the cell suspension is made are all without amyloid, no amyloid emboli are found in the lungs of the recipients

For the succesful induction of amyloidosis in the present transfer experiments several requirements have to be fulfilled 1) The number of casein injections given to the donor mice has to be at least 14 2) An additional requirement is that at least some of the donor spleens contributing to the cell suspension must be in the amyloid phase 3) The duration of the nitrogen mustard treatment plays an important role for the development of amyloidosis In the present experiments the duration varies from none to seven days Amyloid is not detetable until 48 hours after the cell transfer The results show that the longer the treatment, the more frequently does amyloidosis occur in the recipients

SUMMARY

Amyloidosis has been induced experimentally in C3H mice by the transplantation of spleen cells to healthy syngeneic recipients The transferred cells derive from mice hyperimmunized with casein, after intravenous inoculation of the cells the recipient mice are treated with nitrogen mustard The recipients then regularly develop spleen amyloidosis in the course of three to six days Transfer of spleen cells from normal mice does not lead to amyloidosis in the nitrogen mustard-treated recipients, neither does nitrogen mustard treatment alone

Evidence collected from the literature concerning the fate of intravenously transferred cells and the histological peculiarities of the amyloid found in our recipients are indications that the amyloid is formed in the same areas as are colonized by the greatest number of

transferred cells The tentative conclusion is that the amyloid is produced *in loco* by the transferred cells

The two consecutive influences brought to bear on the transferred cells and leading to amyloidosis in the recipients prolonged antigenic stimulation in the donor mouse, suppression by nitrogen mustard in the recipient mouse, support the theory of a local cellular and biphasic genesis of amyloidosis

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PHAGOCYTOSIS IN EXPERIMENTAL MOUSE AMYLOIDOSIS

By

POUL RANLØV

Received 14 iii 66

The essential rôle of mesenchymal cells in the development of experimental amyloidosis has been stressed by Teilum in a number of histological studies during the last two decades His theory of a cellular and biphasic pathogenesis of amyloid disease has recently been reviewed and summarized (*Teilum* 1964) This concept has been supported, among others by the observations that cortisone (*Teilum* 1952), nitrogen mustard (*Teilum* 1954), and x-irradiation (*Christensen & Hjort* 1959) may accelerate the amyloid phase, while thymectomy may accelerate the initial, pyroninophilic phase (*Ranløv* 1966) Additional evidence of the cellular pathogenesis of experimental amyloidosis has recently been produced by the demonstration that transfer of spleen cells from caseinate-treated mice may produce spleen amyloidosis in normal, syngeneic recipients (*Werdelin & Ranløv* 1966) Lately others have been able to demonstrate maintenance of amyloid deposits and P A S positive reticular cells in rabbit spleen explants (*Cohen, Gross & Shirahama* 1965)

In an attempt to elucidate the functional effects of experimentally induced amyloidosis on the immune apparatus we were able to demonstrate a prolonged homograft survival in mice during the amyloid phase (*Ranløv & Jensen* 1966) As a consequense of this observation we found it desirable to investigate other aspects of the immune defense mechanisms during the phasic development of experimentally induced amyloidosis—especially those directly involving the reticular tissues Below an account is given of the phagocytic properties of the R E S during the different phases of caseinate-induced amyloidosis in mice together with some morphological observations concerning the relationship between reticular cells and amyloid deposits

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MATERIAL AND METHODS

The material comprises a total of 82 mice of the inbred ST/a strain All mice were male weighing over 22 gm at the beginning of the experiments (In preliminary investigations younger normal animals were found to exhibit too wide individual variations in phagocytic properties) During caseinate treatment they were fed on oats and tap water They were divided as follows

group N 18 normal non treated controls
group a 18 mice receiving 7 days of caseinate treatment
group b 9 mice treated for 10 days with caseinate
group c 8 mice treated for 14 days with caseinate
group d 7 mice treated for 30 days with caseinate
group e 10 mice treated for 50 days with cascinate and
group f 12 mice treated for 21 days with caseinate

(The original carbon suspension being used up and a new batch containing carbon particles of a different size carbon clearance studies could not be carried out in group f as planned However the group was included in order to define the degree of amyloidosis at this stage of the amyloid induction)

Amyloidosis was induced with sodium caseinate according to the method originally descr bed by *Kuc ynski* (1922) Half a ml of a 5 per cent solution of sodium caseinate in 0 25 per cent NaOH was injected subcutaneously at varying sites of the back 6 days a week

The phagocytic activity was determined by means of the carbon clearance method as described by *Bio i Benacerraf & Halpern* (1953) The carbon used was India ink Pelikan C 11/1431 a obtained from Messrs Günther Wagner Hannover Germany This preparation has been shown not to agglutinate in the blood vessels and to be non toxic (*Halpern Benacerraf & Bio i* 1953) Prior to use it was diluted with a 1 per cent solution of gelatin in sterilized isotonic saline Analysis following digestion of the gelatin with pepsin showed this solution to contain 19 mg of carbon per ml This same solution from the same batch was used throughout the experiments An electron microscopic examinati n showed a grain size below 250 Å for all carbon particles

On the day of the last casein injection each animal was weighed and subsequently injected in the tail vein with one ml of carl on suspension per 100 gm body weight i e 19 mg of carbon per 100 gm mouse The animal was then bled ½ 2 4 6 10 20 and 30 minutes after injection Prior to injection a blind was obtained Blood was drawn alternately from both retroorb tal plexes into heparinized capillary tubes (Clay Adams®) previously calibrated to 0 025 ml with acetone Each blood sample was dissolved in 2 ml 0 1 per cent sodium carbonate and read within 2 hours in a Beckman B spectrophotometer at wavelength 675 nanom The blood concentrations of carbon were expressed as extinction values (extinction 1 000 equivalent to approximately 250 mg per 100 ml whole blood)

2 3 hours after the injection the animals were killed with ether The weight of the liver and spleen was recorded and the following organs were fixed in 10 per cent neutral formalin spleen liver kidney adrenal ilium lung and thymus After embedding in paraffin sections were prepared 5 microns thick and stained with methyl green pyronin periodic acid Schiff at pH 4 1 and occasionally with alkaline Congo red In a certain number of cases sections were treated with diastase f r one hour prior to P A S staining

In 3 cases (group f) half a ml of a 1 per cent solution of Congo red in isot n c sterilized saline adjusted to pH 7 8 was injected intravenously 2 hours prior t killing Tissues from these mice were fixed in Carno s solution and embedded in paraffin avoiding water Sections were cut and deparaffinized and mounted witho t further staining By this means a most brillant birefringence of the amyloid d posits was obta ned when viewed under crossed polars

Amyloid was identified by its morphology and by its birefringence with Congo red The degree of amyloidosis was estimated on sections of the spleen and gra l l from 2 to 6 according to *Christensen & Hjort* (1959)

For each group of animals the mean blood carbon concentrations at ½ 2 4 6 10 20 and 30 minutes respectively were calculated and plotted (Fig 1) For ea h group a curve was obtained demonstrating the carbon clearance as an exponential function of time If plotted as log concentration against time the resulting curves

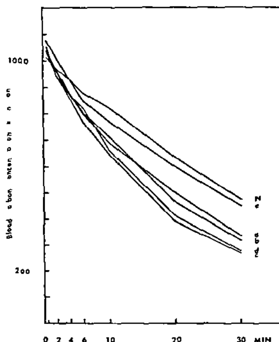


Fig 1

Blood clearance of intravenously administered colloidal carbon (19 mg per 100 gm mouse) in a group of normal mice (N) and groups of mice treated with sodium caseinate for 7 days (a) 10 days (b) 14 days (c) 30 days(d) and for 50 days (e)

became straight lines The phagocytic activity for each group may thus be expressed as the amount of carbon cleared from the blood stream in a certain period of time or according to *Bio i Benacerraf & Halpern* (1953) as $K = \frac{\log C_{t_1} - \log C_{t_2}}{t_2 - t_1}$ K being the granulopectic or phagocytic index In the present material the phagocytic ind ces were calculated from the blood carbon concentrations at 14 and 20 minutes respectively after the carbon injection

Empirically it has been found that individual variat ons in phagocytic indices for a given dose of carbon in normal untreated rats largely depend on differences in the relative weights of liver and spleen This conclusion was supported by the fact that *more than 90 per cent of the injected carbon can be recovered from these organs after* disappearance from the blood stream (*Bio i Benacerraf & Halpern* 1953) Furthermore the phagocytic index (K) seemed to vary with the cube of the relative weights of the organs involved A corrected phagocyt c index was proposed

$$K_{corr} = \frac{\text{body weight}}{\text{liver spleen weight}} \times \sqrt[3]{K}$$

With approx mation this corrected index can be said arbitrarily to represent the activity of a single phagocytizing cell An attempt with all reservations to apply this corrected index (K_{corr}) has been made in the present material

RESULTS

Functional studies Fig 1 shows the carbon clearance curves for each group of normal or casein treated mice Each curve represents mean

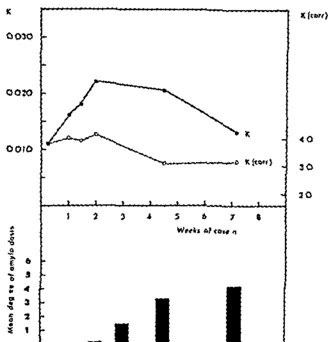


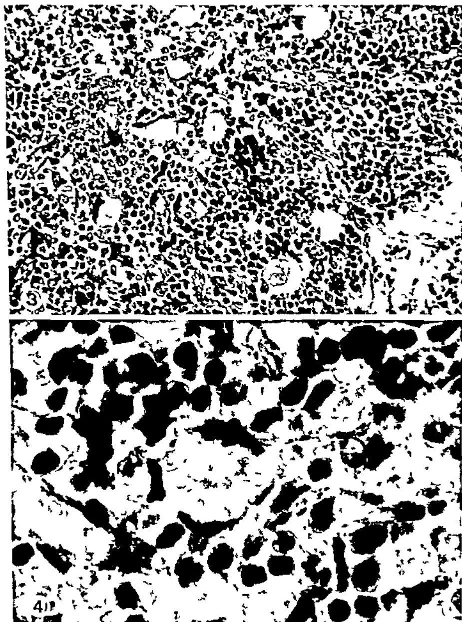
Fig 2

Phagocytic indices (K) and corrected phagocytic indices (K_{corr}) in normal ST/a mice and groups of mice treated for various lengths of time with sodium caseinate Compared with the mean degree of splenic amyloidosis in the various groups

values of blood carbon concentrations at given time intervals for each particular group For the given dose of carbon (19 mg per 100 gm mouse) the clearance in all groups appears to be regular and progressive From Fig 1 a steady increase in the phagocytic activity during the first 2–3 weeks of caseinate treatment is evident After 3–4 weeks of casein the activity tends to decrease and it is almost normal after 7 weeks of treatment This is better illustrated in Fig 2 where the phagocytic indices for each clearance curve have been calculated and plotted against the duration of the caseinate treatment

Tentatively, in order to eliminate the influence of the increasing liver-spleen weights during the caseinate treatment, the corrected phagocytic indices (K_{corr}) have been calculated and plotted in Fig 2 (dotted line) These values seem to follow those of the phagocytic indices, the increase being less pronounced, though However, the values of K_{corr} at 4 and 7 weeks of caseinate treatment ought not to be considered, as the influence of increasing amounts of amyloid in spleen and liver is unpredictable Both curves indicate a decrease in the phagocytic function previous to or synchronous with any significant amyloid formation

Histological studies At gross examination 3 hours after the carbon injection all organs looked macroscopically normal except liver and spleen, which were found coloured entirely black In this respect no



Figs 3–4

Fig 3 (top) Distribution of carbon in the spleen of a mouse treated for 7 weeks with caseinate Note accumulation of carbon particles in the areas of newly formed amyloid (left) At bottom right old amyloid with absence of carbon Periodic acid Schiff × 525

Fig 4 Small newly formed amyloid deposit surrounded by carbon-containing P A S positive reticular cells Note the fibrillar structure of the amyloid Periodic acid Schiff × 840

difference was noted between normal controls and casein-treated mice

At microscopic examination of the lungs, thymus, intestines, and kidneys only occasional carbon granules were found within vessels, reticular or endothelial cells In these respects the findings were identical in controls and in the experimental animals, the carbon being exclusively located in liver and spleen

In the controls most Kupffer cells in the liver were distended with carbon In the spleen, carbon was found uniformly distributed throughout the red pulp with accumulation in the perifollicular zones The particles were mainly confined to the cytoplasm of splenic macrophages In animals having received caseinate injections for 7, 10, and 14 days, respectively, as yet not showing amyloidosis, the distribution of the carbon was somewhat different Though still evenly distributed in the splenic red pulp the accumulation peripherally in the broad perifollicular zones was considerably increased At this moment the pyronimophilia was marked Clusters of pyronimophilic cells were found in the perifollicular collar, some of them being plasmacytoid but the majority being medium-sized or large reticular cells containing pale, round or ovoid nuclei with single dark-staining nucleoli Only a small proportion of these pyronimophilic reticular cells contained phagocytized carbon

After 4 and 7 weeks of caseinate treatment all mice were amyloidotic The amyloid was located mainly in the spleen as broad rings around the splenic white pulp In the liver the amyloid deposits were more sparse and less regularly distributed In P A S stained sections from spleens the age of the amyloid deposits could be estimated from the intensity of its staining, the more central parts of the perifollicular rings staining faintly, the peripheral parts and the small amyloid deposits in the red pulp staining more vividly purple besides showing a more distinct fibrillar structure In the immediate vicinity of the newly formed amyloid deposits reticular cells were found fairly regularly The cytoplasm of these cells was usually ample, finely granulated and in many instances P A S positive, even after previous treatment with diastase Often no clear distinction could be made between the cytoplasm of these cells and the fibrillar structure of the adjacent small amyloid deposits (Fig 4)

The distribution of the carbon particles in the spleens of amyloidotic mice was quite different from that of controls or non-amyloidotic caseinate treated animals The usual localization throughout the splenic red pulp was more sparse and a pronounced accumulation of carbon particles was found in the areas of newly-formed amyloid deposits In contrast, only minute amounts were seen in the more central perifollicular regions of 'old' amyloid (Fig 3) Most carbon was found in the various types of macrophages, but in addition many of the large P A S positive cells contained varying amounts of carbon (Fig 4)

DISCUSSION

In the present investigations yet another function of the R E S—the phagocytosis—was found to vary with the phasic development of experimental amyloidosis the initial pyroninophilic phase was accompanied by a steady increase in phagocytosis while the second, amyloid phase coincided with a decrease in phagocytic activity This corresponds to our earlier findings of a delayed homograft reaction in amyloidotic mice (*Ranlov & Jensen* 1966) The reason for the increased phagocytosis during the first few weeks of caseinate-treatment is not entirely clear The initial hyperplasia of the R E S following the antigenic stimulation, reflecting itself in an increase in the mean liver spleen weight from 1378 mg in the control group to the maximum of 2008 mg in the group receiving 4 weeks of casein, may conveniently explain the increased phagocytic indices as simple mass effect On the other hand, the parallel increase in the 'corrected phagocytic indices' (Fig 2) may indicate an increase in phagocytic property on behalf of the single cell Data suggesting a dependence of serum factors have been put forward Among these opsonins (*Jenkin & Rowley* 1961), complement (*Spiegelberg, Miescher & Benacerraf* 1963), properdin (*Nanni* 1958), and various isoantibodies (*Bennett, Old & Boyse* 1963) Recently *Normann & Benditt* (1965) apparently succeeded in isolating a phagocytosis-promoting factor from rat serum by extraction with barium sulphate Further evidence in favour of a participation of an extracellular factor lies indirectly in the investigations by *Moore et al* (1961) They found in a purely morphological study phagocytosis of saccharated iron oxide mostly confined to cytologically unaltered reticular cells in rabbits previously treated with Freund s adjuvant In contrast, only a few of the many proliferating reticulo endothelial cells accumulated iron in their cytoplasm In view of the fact that the total phagocytic activity in the animal—that is, the clearance from the blood stream of a given amount of injected colloidal material—actually *do* increase during antigenic stimulation, the assumption of an increased activity in the single reticulum cell seems justified In the present material this increase has been found to coincide with the pyroninophilic phase and the subsequent decrease to coincide with the amyloid phase The breakdown of the protein synthetizing function of mesenchymal cells which marks the turning point between the two phases in amyloid production may well involve a simultaneous decrease in the amount of protein like substances promoting phagocytosis of particulate matters

During the accomplishment of the present investigations *Shearing, Comerford & Cohen* (1965) reported their findings in a functional study of the effect of an amyloid inducing regimen on the phagocytosis in rabbits They conclude that the R E S plays an important rôle in the pathogenesis of secondary amyloidosis, involving at least two sequential events R E S hyperplasia and subsequent amyloid for-

mation, thus supporting Teilum's concept of a biphasic, cellular morphogenesis involving mesenchymal tissue They do not report morphological details The morphological findings in the present material do fully support *Smetana's* (1927) observations He found R E S cells with phagocytic properties located exactly in places where amyloid was most constantly seen Based on blocking experiments with rather equivocal results, he reported that amyloid appeared later in animals whose reticulo endothelial cells were filled with India ink *Christensen* (1966), however, found increased development of amyloidosis in caseinate-treated mice after blocking of the R E S with Thorotrast He ascribes this to the radiation effect of the injected colloid

Kennedy (1962) objects to the cellular theory of local secretion mainly because he finds no definite topographical relationship between amyloid deposits and plasma cells He does not consider the reticulum cell a probable source of amyloid substances, mostly because of *Marshall's* statement (1956) that globulin synthesis is not a function of cells with phagocytic properties However, Marshall's views in these matters have been questioned by several investigators In electron microscopic investigations ergastoplasm has been demonstrated in the cytoplasm of metalophilic reticulum cells (*Stoeckenius & Naumann* 1957) and, likewise ergastoplasm and increased RNA has been found in pulmonary macrophages (*Karrer* 1960) *Rebuck* (1961) found lymphocytes in the inflammatory exudate surprisingly phagocytic after a very brief sojourn at the inflammatory site, he claims a transition into macrophages *Granger & Weiser* (1966) succeeded in isolating a specific haemagglutinin from well washed immune peritoneal macrophages, thus suggesting some kind of biosynthetic activity in this cell-type An *in vitro* transformation of lymphocytes into macrophage-type cells in the liver during the graft-versus-host reaction has recently been described by *Howard* (1966) *Gowans* (1962) showed the transformation of labelled small lymphocytes into large, pyroninophilic reticular cells

Thus a potential interchange between functionally different types of mesenchymal cells seems to have been substantiated The 'classic anti body-producing cells—the plasma cells and the lymphocytes—may not necessarily monopolize protein synthesis, especially not during pro longed antigenic stimulation as is the case in the caseinate treatment leading to amyloidosis Serum protein changes during such treatment are usually initiated by an increase in the gamma fraction During pro longed treatment a rise in the beta and alpha fractions ensue (*Bohle Hartmann & Pola* 1950, *Teilum, Harboe & Lieck* 1953, *Christensen & Rask-Nielsen* 1962, *Ranlov* 1966) This dislocation of protein synthesis towards more low molecular types of protein may well reflect the increasing dedifferentiation of mesenchymal tissue cells towards more primitive reticular cell-types eventually resulting in a functional breakdown with subsequent deposition of abnormal glycoproteins in the tissues

SUMMARY

A functional study of the blood clearance of injected colloidal carbon was undertaken in order to investigate the effects of prolonged amyloidogenic stimulation on the R F S in several groups of mice treated with sodium caseinate for different lengths of time

It was found that phagocytosis varied with the phasic development of experimental amyloidosis the initial pyroninophilic phase being accompanied by a steady increase, the second, amyloid phase coinciding with a decrease in phagocytic activity thus supporting Teilum s concept of a biphasic cellular pathogenesis of amyloidosis

The morphologic studies showed phagocytosis predominantly to occur in the border zones of the amyloid deposits and most markedly in areas of newly formed amyloid thus demonstrating the intimate relationship between reticular tissues and amyloid

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THE INHIBITORY EFFECT OF MOUSE GAMMA GLOBULIN ON THE LYSIS OF ISOLOGOUS MOUSE TUMOUR CELLS BY HUMAN SERUM

By

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Received 9 II 66

Cells of the Bergen A4 ascites carcinoma in common with many other mouse tumour cells (18 24, 19 1, 20 7), undergo lysis when they come into contact with fresh human serum (8) and it appears that complement is essential to the process (9) These tumour cells have also been shown to lyse in the peritoneal fluid from isologous mice (10) Thus mouse complement as well as human and many other types of complement (19, 13), seems to be able to mediate this lytic reaction (10)

It has also been shown that the ascitic fluid from this tumour inhibits the reaction when heterologous complement is used (11), as does the serum of isologous mice (10, 13 14) As this latter serum can also be shown to contain complement (10) it can be concluded that the inhibitory factor(s) is active against isologous as well as heterologous complement

Previous findings, in particular that its action can be abrogated by albumin (14), suggested that the inhibitory factor(s) might be a gamma globulin The present work is an example of experiments set up to test this hypothesis

MATERIAL AND METHODS

Tumour cells from two 14 day transplants of the Bergen A4 ascites carcinoma grown in strain A female mice were used A one in twenty suspension of whole tumour ascites in physiological saline was made up just before use

Human serum from blood donors was used This had been collected and stored as described previously (9)

Tumour ascitic fluid from 11 day transplants of the Bergen A4 ascites carcinoma in strain A male mice was used This was the supernatant obtained after centrifuging the whole tumour ascites The fluid was stored at —20° C Deep freezing caused the fibrinogen to separate out The resultant clot was discarded The remaining fluid contained albumin and alpha beta and gamma globulin (see Table 1)

Protein fractions were prepared from this fluid by alcohol fractionation by Cohn s method (2) The resultant fractions were dried in vacuo and resuspended in physiological saline The following fractions were used that containing alpha and

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beta globulin that containing alpha and beta globulin plus albumin and that containing alpha beta and gamma globulin

The total protein content of the fractions was determined spectrophotometrically by the biuret method and the distribution of the different proteins in the fractions by paper electrophoresis The composition of the fractions is detailed in Table 1

TABLE 1

The Protein Content of the Test Fluids i e Tumour Ascitic Fluid Fractions and Whole Tumour Ascitic Fluid

Test fluid		Protein content (g%)			
			globulin		
No	Type	albumin	alpha	beta	gamma
1	Fraction†	0 0	1 4	3 1	0 0
2	Fraction	2 2	0 7	0 4	0 0
3	Fraction	0 0	1 7	1 5	0 4
4	Ascitic fluid	1 5	0 2	0 8	0 3

† Resuspended in physiological saline

Preparatory Tests

The test fluids—i e the tumour ascitic fluid and the resuspended protein fractions—were first checked for possible toxic action on the tumour cells One drop of each tumour cell suspension in turn was added to one drop of the test fluid plus one drop of physiological saline Control cells were added to two drops of saline Wet preparations were made and examined for oncolysis as described previously (9) after 30 minutes at 20° C The degree of lysis was recorded as absent incomplete (some lysed cells present) and complete

The human sera were then tested for lytic activity One drop of each of the tumour cell suspensions in turn was added to one drop of serum from each of 9 blood donors in turn plus one drop of saline Wet preparations were prepared and examined as above

Experimental Procedure

The action of the test fluids on the lysis of *Bergen 44* ascites carcinoma cells by human serum was tested as follows

One drop of each of the test fluids in turn and also for comparison one drop of saline was added to one drop of whole serum One drop of tumour cell suspension was then added to each tube wet preparations made as described above and examined for tumour cell lysis every 5 minutes for 30 minutes

The tests were repeated with another serum and the same tumour cell suspension and with another serum and the second tumour cell suspension The whole experiment was then repeated using a one in one dilution of human serum in physiological saline in place of whole serum in each case

RESULTS

Preparatory Tests

Test fluids No lysis occurred in any of the preparations It was therefore concluded that the test fluids in themselves were not toxic to the tumour cells and that the viability of the tumour cells was not in question

Human sera Six of the sera gave complete lysis within 30 minutes With the other three lysis was incomplete Three of the highly lytic sera were chosen for further use

Experimental Work

The action of the test fluids on lysis by human serum

The results with *whole serum* are shown in Table 2 They were similar in all three tests—*i e* with the two different tumour cell suspensions and the three different sera

TABLE 2

The Effect of the Different Test Fluids i e Tumour Ascitic Fluid§ and Saline on the Oncolysis of Bergen A4 Ascites Carcinoma Cells from two Different Transplants by three Human Sera at 20° C*

Reactants			Degree of lysis† at (mins)						
Cells	Serum	Test fluid	0	5	10	15	20	25	30
1st trans plant	1	saline	0	++	++	++	++	++	++
		1	0	+	++	++	++	++	++
		2	0	+	++	++	++	++	++
		3	0	0	+	+	+	+	+
		4	0	0	+	+	+	+	+
	2	saline	0	+	++	++	++	++	++
		1	0	+	++	++	++	++	++
		2	0	+	++	++	++	++	++
		3	0	+	+	+	+	+	+
		4	0	+	+	+	+	+	+
2nd trans plant	3	saline	+	+	++	++	++	++	++
		1	0	+	++	++	++	++	++
		2	0	+	++	++	++	++	++
		3	0	+	+	+	+	+	+
		4	0	+	+	+	+	+	+

† 0 = absent
\+ = incomplete
\++ = complete

§ for protein content see Table 1
* fractionated (1 2 3) and whole (4)

In the combination cells serum-saline complete lysis occurred within 5 to 10 minutes The action of the fluid containing only alpha and beta globulin and that containing alpha and beta globulin plus albumin was similar, and differed little from that of saline—lysis being complete within 10 minutes On the other hand the action of the fluid containing gamma, in addition to alpha and beta globulin was indistinguishable from that of the unfractionated ascitic fluid which contained all three globulins plus albumin Both these fluids had a marked inhibitory effect on the reaction compared to saline Though some tumour cells had lysed in both cases by 10 minutes the process stopped there Complete lysis was not seen even after 30 minutes

The results in *serum dilution* are shown in Table 3 The reaction was slowed up compared to that in whole serum but otherwise the results were in essence similar in that the inhibitory action was found to lie in the fraction containing gamma globulin This fraction was slightly less inhibitory than the unfractionated fluid, lysis appearing from 5 to 10 minutes earlier in the latter In two cases lysis occurred slightly

earlier with the fraction that contained alpha and beta globulin plus albumin than with that containing alpha and beta globulin alone In the third no difference was apparent

TABLE 3

The Effect of the Different Test Fluids i e Tumour Ascitic Fluid § and Saline on the Oncolysis of Bergen A4 Ascites Carcinoma Cells from two Different Transplants by three Human Sera Diluted 1 1 with Saline at 20° C

Reactants			Degree of lysis at (mins)†						
Cells	Serum	Test fluid	0	5	10	15	20	25	30
1st trans plant	1	saline	0	+	++	++	++	++	++
		1	0	+	+	+	++	++	++
		2	0	+	+	++	++	++	++
		3	0	0	0	+	+	+	+
		4	0	0	0	0	0	+	+
	2	saline	0	+	+	+	+	++	++
		1	0	+	+	+	+	++	++
		2	0	+	+	+	++	++	++
		3	0	+	+	+	+	+	+
		4	0	0	+	+	+	+	+
2nd trans plant	3	saline	0	+	+	+	++	++	++
		1	0	+	+	+	++	++	++
		2	0	+	+	+	++	++	++
		3	0	0	+	+	+	+	+
		4	0	0	0	+	+	+	+

† 0 = absent
+ = incomplete
++ = complete

§ for protein content see Table 1
fractionated (1 - 3) and whole (4)

DISCUSSION

The present experiments show that the factor(s) responsible for the inhibitory effect of tumour ascitic fluid on the lytic action of fresh human serum on Bergen A4 ascites carcinoma cells resides in its gamma globulin fraction

The experiments confirm that human serum whole and in dilution has a marked lytic effect on these tumour cells (12) and that this effect is strikingly reduced *in vitro* in the presence of tumour ascitic fluid (11) The ascitic fluid used here had been deep frozen and the fibrinogen had precipitated out (see above) Even so it exerted a similar inhibitory effect to that seen with fresh tumour ascitic fluid (11) This rules out the possibility that fibrinogen is the inhibitory factor The test fluid containing alpha and beta globulin on[illegible] lacked inhibitory action as did that contain[illegible] plus albumin The fl[illegible] globulin was howev[illegible] already shown that alpha a[illegible] without action in this respect these results indicate that the inhibitor of oncolysis lies in the gamma globulin fraction of the tumour ascitic fluid

The explanation may be that the gamma globulin in the tumour ascitic fluid is anticomplementary The concept that gamma globulin may be anticomplementary is not new It was first suggested in 1911 that *the globulin fraction of stored serum might be anticomplementary* (25), and in 1944 isolated human gamma globulin was shown to be so, its action being masked in whole serum by that of albumin (5) This latter finding accounts for the frequent appearance of anticomplementary activity in Cohn's fraction II which contains the gamma globulins In the present experiment the presumed anticomplementary activity was present in the tumour ascitic fluid before fractionation, and later confined to the test fluid containing gamma globulin However, the fractionated sample contained more gamma globulin (0 4 g%) than the unfractionated (0 3 g%), which also contained albumin Even so, when the oncolytic reaction was slowed down by using the human serum in dilution (12), it was evident that the fractionated sample was slightly less inhibitory than the unfractionated Thus activity has been *lost rather than gained on fractionation It is therefore unlikely that* the inhibitory effect of the test fluid is an artefact due to fractionation Further, the fraction containing albumin in addition to alpha and beta globulin was slightly more lytic than that containing alpha and beta globulin alone, suggesting that mouse albumin, as well as bovine albumin (15), may potentiate oncolysis The idea that the gamma globulin in the tumour ascitic fluid may act via an anticomplementary effect is supported by the finding that its action can be abrogated by albumin, both *in vitro* (14) and possibly *in vivo* too (16)

Anticomplementary action has in the past been regarded as a laboratory hazard—a nuisance to be avoided To quote *Coombs et al* (3), ' For practical purposes it is convenient to consider anticomplementary effects as weeds, in the horticultural sense, in that they are unwanted ' An exception is to be found in the anticomplementary action of sera containing the rheumatoid factor, itself a gamma globulin, which led to the discovery of the factor (23), and thus serves as an example of the usefulness of such action *in vitro* The present findings indicate that the anticomplementary action of gamma globulin may be of even wider significance Such action may be of importance *in vivo* However, before discussing this possibility it is necessary to analyse the oncolytic reaction itself

In the present work oncolysis was brought about by human serum It is generally agreed that complement is essential to this reaction, and that a further factor, *i e* antibody, is needed to initiate it (see 9) It can be argued that the antibody involved is a heterophil antibody present in the human serum but the possibility that the tumour cells may have been presensitized *in vivo*—*i e* before they were added to the system (9, 10, 12)—can not be excluded If so complement alone will suffice to lyse the tumour cells When pure complement components are available this question may perhaps be settled

On the other hand antibody may not be needed It is known that non-specific factors may on occasion mimic sensitization of erythrocytes—rendering them liable to lyse in the presence of complement (21) Similar factors may operate with tumour cells In the present case either sensitization or pseudosensitization may be at work, and at present they can not be distinguished one from the other However, whether antibody is present or not the tumour cells behave like sensitized cells in that they lyse in the presence of complement This happens both *in vitro* (14) and *in vivo* (22)

When Bergen A4 ascites carcinoma cells are injected intraperitoneally a certain proportion of them lyse in the peritoneal fluid (22) Soon however the presence of these cells calls forth an inflammatory exudate—*i e* tumour ascitic fluid Once exudate formation is established progressive tumour growth ensues It seems that the inhibitory factor(s) in the tumour ascitic fluid protects them from the action of their own complement These findings are in keeping with *Klein & Revesz's* (17) hypothesis that with ascitic tumours, "cell proliferation starts only after a definite critical concentration of some active substance has been built up within the peritoneal fluid" *Klein & Revesz* suggest that the active substance is a product of the tumour cells However the inhibitory factor in tumour ascitic fluid is also present in the serum of non-tumour bearing mice (13, 14) Thus it can not be a product of the tumour cells, but is a normal constituent of mouse body fluids The amount of this substance in the peritoneal fluid must normally be low but should increase when an inflammatory exudate is formed Gamma globulin fits these requirements

Although mouse gamma globulin, *i e* mouse serum, has this inhibitory effect on the action of complement on mouse tumour cells, the sera of other animals, *e g* goat, rabbit and guinea pig, does not (13) Thus there appears to be a species specificity between the gamma globulin and the tumour cell and or its sensitizing antibody The type of complement is not critical (13) As tumour growth is also species specific it seems that the mouse has an inbuilt mechanism that protects its own cells from lysis by complement should they become sensitized, while the serum of animals in which the tumour fails to grow does not have this protective effect In other words the mouse's *own* gamma globulin protects its "*own*" tumour cells from death due to immunological lysis by its *own* complement

Such a protective mechanism may not be confined to ascitic tumour growth in mice If their own gamma globulin can protect sensitized or pseudosensitized tumour cells from immunological damage *in vivo*, the possibility that other cells that have by chance become antigenic may be protected from damage in a similar way needs to be explored This could explain the frequent occurrence of circulating auto-antibody in the absence of autoimmune disease which would then occur only if there were a concommitent defect in gamma globulin synthesis, *i e* a

lack of normal gamma globulin There is evidence that autoimmune disease is frequent in patients with hypogammaglobulinaemia (4) Similarly autoimmune disease may occur in dysgammaglobulinaemia (4), in which paradoxically, the actual amount of abnormal gamma globulin may be high, as it is in NZB mice (6) These two associations support the hypothesis that normal gamma globulin in sufficient amount may be necessary to prevent tissue damage by auto antibody In other words the gamma globulins may play their part in the maintenance of immunological homeostasis If so gamma compatibility may be of importance in homotransplantation Indeed if one follows this argument to its logical conclusion one is forced to ask, 'Is the anti complementary action of gamma globulin a part of the mechanism that permits the survival of self in an immunologically competent organism?'

SUMMARY

The factor(s) in tumour ascitic fluid that inhibits the lytic action of human serum on Bergen A4 mouse ascitic tumour cells is shown to lie in its gamma globulin fraction It is suggested that its action may be anticomplementary The question as to whether similar anticomplementary action of gamma globulin may facilitate tumour growth *in vivo* is raised and it is pointed out that its effect seems to be species specific Evidence that such a mechanism may also play its part in the maintenance of immunological homeostasis is discussed

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THE HOST RESPONSE OF IRRADIATED MICE TO EHRLICH'S ASCITES CARCINOMA

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The influence of host immune response on the intraperitoneal growth of Ehrlich's ascites carcinoma (EAC) has been discussed previously (*Hartveit* 1961, *Wheatley & Ambrose* 1964) As this tumour arose in a non inbred strain all transplants in mice may be regarded as homografts, but unlike other homografts this tumour grows and usually kills its host

The wide homotransplantability of this and other non specific tumours has been attributed to various factors A positive correlation in these tumours between tetraploidy and ability to grow in foreign strains was shown by *Hauschka & Levan* (1953), and a current explanation of homotransplantability is decreased isoantigenicity of the tumour cells due to chromosomal changes (*Hauschka et al* 1956) In contrast, *Feldman & Sachs* (1957) found that homotransplantability could be correlated to increased antibody production In addition, it has also been shown that some non specific tumours grow in spite of histocompatibility—2 (H 2) incompatibility, though this has not been demonstrated in the case of EAC (*Hauschka & Amos* 1957)

Hartveit (1963) has found increased stainability, clumping, fine intercellular filaments and swollen tumour cells with cytoplasmatic damage during growth of EAC These changes have been taken as an expression of host immune response The facts that EAC cells absorb complement and consequently show lysis like sensitized sheep red cells when human serum is added (*Hartveit* 1965 a), and that heterophil antibodies, though not definitely excluded, are unlikely to be responsible for these reactions (*Hartveit* 1965 b) give further evidence that EAC cells become sensitized or antibody coated during intraperitoneal growth

On the whole these findings indicate that homotransplantable tumours elicit an immune response and that they grow in spite of this

The present experiments were set up to test the role of this presumed host immune response on the cytology and intraperitoneal growth of EAC Total body irradiation was used to modify this re-

sponse and an X-ray dose of 300 r was chosen because it has been shown to be sufficient to depress or delay antibody response to bacterial agents (*Makinodan & Gengozian* 1958, *Silverman & Chin* 1955) and to homografted tumours (*Werder et al* 1953) A preliminary study showed that 300 r given to mice of our closed colony was consistantly nonlethal and gave no weight loss This was considered of importance in the present experiments, as it is known that higher doses of whole body irradiation cause considerable nutritional alteration and weight loss This may influence the establishment and growth of transplanted tumours (*Tannenbaum* 1959) A futher preliminary study to check the effect of this radiation dose on the rejection of a strain A specific tumour in our closed colony was also included

MATERIAL AND METHODS

Mice Mice from the inbred strain A/Sn originally obtained from Professor Klein at Karolinska Institutet in Sweden and from an unrelated out bred but closed colony kept at our Institute were used The experiments were carried out with male and female mice 2 to 3 months of age The animals were kept on a standard pellet diet Food and drinking water were available ad libitum

Tumours TA_3 obtained recently in ascitic form from Professor Klein originated as a spontaneous mammary adenocarcinoma in a female A mouse and grows progressively in strain A mice and AF_1 hybrids (*Klein* 1951) It has been maintained at our Institute by both intraperitoneal and subcutaneous transfers in strain A/Sn mice and AF_1 hybrids It is rejected by our closed colony

The Ehrlich ascites tumour near tetraploid line was placed at our disposal in 1959 by Professor Ahlstrom Lund in Sweden where it originally came from Professor Klein It has been maintained by serial ascitic transfer in closed colony mice and at the start of these experiments the tumour was in its 190th transplant generation here

Irradiation The mice were irradiated in a plastic box by means of a Mueller RT 200 machine Irradiation factors were 200 kv 15 mA 46 cm FSD (focus skin distance) and 1 mm Cu HVL (half value layer) The irradiation time was 4 minutes and 6 seconds and the animals received 300 r

Experimental Procedure

Preliminary test of radiation effect on the immune response One group of 5 male mice (mean weight 23 0 g) and 5 female mice (mean weight 21 0 g) from strain A/Sn and two groups of 5 males (mean weight 23 4 ± 0 5 g) and 5 females (mean weight 23 2 ± 1 0 g) from the closed colony were set up TA_3 (4×10^6 cells) prepared in sterile 0 85 percent saline from a 25 day old subcutaneous transplant in a female A/Sn mouse were given subcutaneously to the A/Sn mice to one group of the closed colony mice irradiated 24 hours previously and to one group of sham irradiated closed colony mice The two greatest tumour diameters at right angles were measured every 2nd day The sum of these values divided by 2 was recorded and means calculated for each group

Radiation effect on cytology and intraperitoneal growth of EAC Two groups of 15 male closed colony mice were used the mean weight was 22 9 ± 1 4 g One group received irradiation while the other was sham irradiated Twenty four hours after irradiation the mice in both groups were each given an intraperitoneal injection of 0 1 ml of tumour ascites from a 9 day old transplant in a male mouse The tumour cell count in the inoculum was 13×10^6

A third group of 10 male mice was given irradiation only as a further control of the radiation effect on general health and survival

The two groups of 15 mice were killed by cervical dislocation 7 days following

tumour injection Films were made immediately from the tumour ascites air dried and stained with Leishman s stain The morphology of the tumour cells was studied Two hundred cells were counted and the number of large injured tumour cells and non tumour cells (minus erythrocytes) expressed as a percentage for each mouse

The total volume of the tumour ascites was measured The packed cell volume (PCV) of tumour cells and erythrocytes and the amount of cell free fluid were determined as percentages of 1 ml tumour ascites after centrifuging this in a Wintrobe tube at 1100 g for 45 minutes During this procedure the erythrocytes sediment at the bottom of the tube From these data the total values for packed tumour cells erythrocytes and cell free fluid in ml were determined

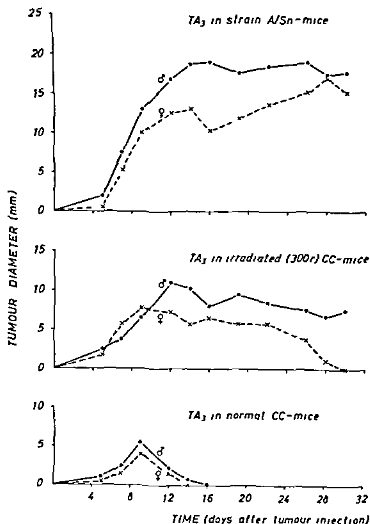


Fig 1

The mean growth curve obtained following subcutaneous injection of a strain A tumour (TA_3) in normal strain A/Sn mice (top) in closed colony mice (CC) total body irradiated 24 hours before with 300 r (centre) and in normal closed colony mice (bottom) Five male and 5 female mice in each group

RESULTS

Preliminary Test of Radiation Effect on the Immune Response

The results are given in Fig 1, which shows that transplantation of TA_3 to the strain of origin gave rise to progressively growing tumours On homologous transplantation to irradiated closed colony mice there is initially progressive growth after which there is a clear tendency for the tumours to persist After the 29th day no tumours were observed in the female mice, while tumours could still be seen in the males

In the normal closed colony recipients tumours were observed which reached their maximum within 9 days Thereafter regression rapidly occured and no tumours were seen after 14 16 days following transplantation

Thus 300 r delays the rejection of this tumour in mice of our closed colony indicating that their immune response to the tumour tissue has been markedly depressed

Radiation Effect on Cytology and Intraperitoneal Growth of EAC

The tumour cells in the non irradiated mice were darkly stained, shrunken, angular and multiple fine intercellular bridges and clumping could be seen (Figs 2 and 3 a) Four per cent large definitely injured tumour cells were present These cells were swollen with cytoplasmatic damage but the nucleus and the nucleoli, though swollen, were preserved (Fig 3 b) Nine per cent of the cells (excluding erythrocytes) in the tumour fluid were of host origin Lymphocytes and large mononuclears predominated

In marked contrast to these findings the majority of the cells in the irradiated mice were light stained and spherical Intercellular bridges could not be demonstrated (Fig 4) Fourteen per cent large injured tumour cells with simultaneous destruction of cytoplasm and nucleus with loss of nucleoli were seen (Fig 5) In other words, these cells were morphological different from the large injured tumour cells found in the non irradiated mice The tumour fluid of the irradiated mice contained 8 per cent non tumour cells, mainly lymphocytes and larger mononuclears, red blood cells excluded

In Table 1 the mean total PCV of tumour cells and erythrocytes and the fluid volume with standard deviation are recorded For statistical evaluation 'Student s' t tests were used (*Snedecor* 1956) The total PCV of erythrocytes was significantly increased in the irradiated mice ($P < 0.05$) Both the PCV of tumour cells and the fluid volume were slightly reduced in the irradiated mice but no significant differences between the groups could be found

The third group of 10 mice given irradiation only showed no weight loss and there were no deaths during the experimental period

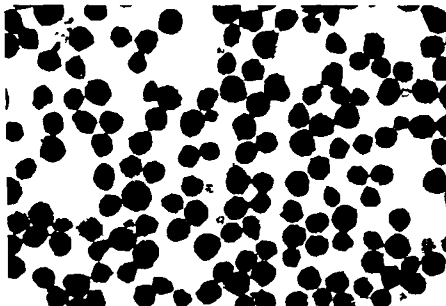
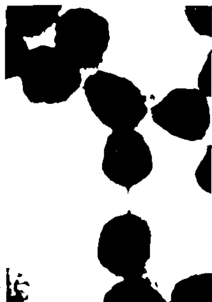


Fig 2

Tumour cells from an untreated control mouse The cells stain darkly and are shrunken angular and show clumping (Leishman s stain × 370)





a

b

Fig 3

Greater magnification of tumour cells from an untreated control mouse (× 1500)

a Tumour cells fine intercellular bridges

b Large lytic tumour cell with cytoplasmatic disintegration blebbing and pre served nucleus and nucleoli

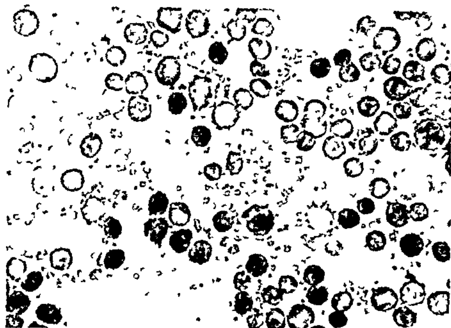


Fig 4

Tumour cells from an irradiated mouse The cells stain lightly No angularity or intercellular bridges present All degrees of lytic damage and a number of red blood cells can be seen (Leishman s stain × 370)



Fig 5

Greater magnification of tumour cells from an irradiated mouse Lytic cells with different degrees of both cytoplasmatic and nuclear damage (× 1500)

TABLE 1

The Total Packed Cell Volume (PCV) of Tumour Cells and Red Cells and the Cell Free Fluid Volume in Irradiated and Control Male Mice 7 Days after Intraperitoneal Injection of Ehrlich's Ascites Carcinoma Entries means (ml) ± SD

Groups	No and sex of animals	PCV of tumour cells (ml)	PCV of red cells (ml)	Cell free fluid (ml)
Irradiated	15 ♂ *	1 16 ± 0 35	0 14 ± 0 09	2 37 ± 0 67
Controls	15 ♂ §	1 21 ± 0 63	0 08 ± 0 09	3 12 ± 1.28

* 1 lost due to technical error
§ 2 lost due to technical error

DISCUSSION

Following transplantation to untreated mice of our closed colony the cells of EAC undergo characteristic changes (*Hartveit* 1963) In early biopsies the majority of the tumour cells are spherical and light stained and some large cells with injured cytoplasm but preserved nucleus and nucleoli are present The number of these large injured tumour cells increases during the first phases of tumour growth Later a marked change occurs in the cytology of the tumour There is a sharp drop in the number of large injured cells while the majority of the tumour cells are smaller and show increased stainability, angularity and clumping Some fine intercellular bridges can also be seen In terminal phases this cytological picture is basicly unchanged but an increasing number of large injured tumour cells can be seen These cells are clearly different from the large injured cells found in the early period mentioned above as they show simultaneous destruction of both cyto plasm nucleus and nucleoli

In the present experiment the tumour cells in the non irradiated mice showed general increased stainability, intercellular bridges and clumping These changes similar to those found in late transplants (*Hartveit* 1963) have been taken as a morphological expression of in vivo sensitization or antibody-coating of the tumour cells due to host immune response, as tumour cells from late transplants are able to absorb complement and lyse when human serum is added (*Hartveit* 1963 a) The immunological nature of these cytological changes may be supported by the fact that similar clumping and intercellular fila ments can be seen in red cells in some cases of autoimmune anaemia (*Dacie* 1962) The large injured tumour cells found in 4 per cent in these mice are identical to those consistently found in this tumour and this damage is presumably of immunological type (*Hartveit* 1962) Similar tumour cell damage can be seen when specific immune serum plus complement are added to EAC cells (*Flax* 1956 *Lindner* 1960)

In marked contrast to these findings the majority of the tumour cells in the irradiated mice were light stained without signs of clump-

ing or intercellular bridges, a picture similar to that seen in early stages of tumour growth in untreated mice Tumour cells from early transplants in untreated mice have also been shown to be less capable of absorbing complement from human serum than cells from late transplants (*Hartveit* 1965 b) Large numbers of injured tumour cells were found in irradiated mice These cells, clearly different from the damaged cells seen in the untreated group, showed the same kind of injury as has been demonstrated in late or terminal transplants and in autolytic tumours This injury has been attributed to anoxia (*Hartveit* 1962)

The increased blood content found in the irradiated mice is probably due to the well known tendency of total body irradiation to produce a haemorrhagic diathesis (see *Upton* 1955) It is reasonable to suggest that the anoxic cells damage found in these mice is due to the increased blood content of the tumours, as such gross bleeding will render the mouse anaemic Products from lytic cells may again damage the capillary endothelium and increase the vascular permeability (*Thunold* 1965) and a vicious circle may be set up

Thus the tumour cell morphology and presence of large injured tumour cells are probably evidence of host immune response in the non-irradiated mice The tumour cells in the irradiated mice showed anoxic damage, while cytological changes indicating an immune response were lacking These conclusions are supported by the results of the preliminary test of the effect of 300 r on the immune response The temporary growth of TA_3 in previously untreated mice of the closed colony followed by complete tumour regression, is the growth pattern normally found after homotransplantation of tumours The increased tumour growth and prolonged survival of the transplants in the irradiated mice indicate that the normal immune response in the closed colony has been abrogated or depressed

The present experiment further shows that the amount of tumour and cell free fluid, which probably is a part of the inflammatory reaction to the tumour cells (*Revesz* 1955, *Hartveit* 1965 c) were the same in the two groups As there were no significant differences in number of non tumour cells (red cells excluded) in the peritoneal fluid of irradiated and non-irradiated mice, the PCV of the tumour cells could be used for comparing tumour growth A few reports on the intraperitoneal growth of EAC in irradiated mice can be found but none in which this growth has been correlated to cytological investigations In contrast to the present findings *Mazurek & Duplan* (1959) demonstrated reduced total ascitic fluid and total number of tumour cells in irradiated mice on the 7th transplantation day *Wheatlay & Easty* (1964) have investigated the growth of EAC in irradiated mice at regular intervals following tumour inoculation They found retarded tumour growth in late and terminal transplants However from their data no obvious differences in amount of tumour can be

seen on the 7th transplantation day and their results are thus not necessarily contrary to those presented in this experiment

As the total amount of tumour cells, in contrast to the cytological findings was similar in the irradiated and non irradiated mice in the present experiment, it can be concluded that the absence or presence of the supposed host immune response was of no consequence to intra peritoneal tumour development under these conditions

SUMMARY

The intraperitoneal growth of Ehrlich's ascites carcinoma has been investigated 7 days following transplantation in normal and previously irradiated (300 r) closed colony mice A preliminary experiment with a strain A specific tumour showed that this X ray dose was sufficient to permit its growth in the unrelated closed colony mice

The cytological findings support the previous suggestion from this Institute that the tumour cells become antibody coated during growth in normal mice In the irradiated mice such cytological changes indicative of an immune response were not found

The amount of blood in the tumour ascites was increased in the ir radiated mice probably due to irradiation effect

The amount of tumour and cell free fluid were similar in the two groups

It is concluded that the immune response of the recipients is of no consequence to the intraperitoneal growth of EAC under these condi tions

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THE SURVIVAL TIME OF IRRADIATED MICE WITH EHRLICH'S ASCITES CARCINOMA

By

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Received 16 ii 66

In a recent work the intraperitoneal growth of Ehrlich s ascites car cinoma (EAC) was investigated in irradiated and normal mice (*Thunold* In press) Tumour cell changes indicative of host immune response were observed in the normal mice but not in the irradiated animals Irradiation of the hosts did not appear to affect tumour growth significantly but seven days after transplantation the irradiated mice showed a significant increase in tumour blood content

The following investigation on the influence of irradiation on survival time of tumour bearing mice was planned as a consequence of these findings as a negative correlation has been demonstrated between the amount of blood in the ascitic fluid and survival time after transplan tation of EAC to previously untreated mice (*Hartveit* 1961)

MATERIAL AND METHODS

Mice These were similar to the closed colony mice used in the previous experiment Males and females were of the same age and two groups of 15 males (mean weight 22 8 ± 0 8 g) and 15 females (mean weight 20 5 ± 1 3 g) were set up

Tumour The Ehrlich ascites carcinoma used was in its 239th transplant generation here and an 8 day transplant from a female mouse was used All animals received 13 × 10^6 tumour cells in 0 1 ml of whole tumour ascites intraperitoneally

Irradiation Whole body irradiation was given 24 hours prior to tumour inocula t on to one group of 15 males and 15 females The control group was sham irradiated Irradiation factors were as in the previous experiment (200 kv 15 mA 46 cm FSD 1 mm Cu HVL) and the animals received 300 r which has been shown to be suf ficient to depress the immune response in mice of the strain used in this experiment

Another group of 5 males and 5 females received irrad ation only

The mice were weighed every 2nd day and their survival time was recorded in days The total tumour ascites was measured and one ml of the ascites centrifuged The packed cell volume (PCV) of the tumour cells and the erythrocytes and the cell free fluid volume were recorded as percentages of 1 ml of tumour ascites and the total values in ml were calculated as in the previous experiment (*Thunold* In press) The presence and extent of solid tumour growth in the peritoneal tissues were noted

RESULTS

Body weight curves The mean increase in body weight in tumour bearing mice is shown in Fig 1 It can be seen that this increase is

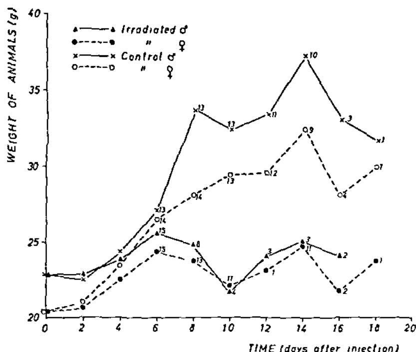


Fig 1

The mean increase in body weight of irradiated and control male and female mice after intraperitoneal injection of Ehrlich's ascites carcinoma The figures represent surviving animals

similar in untreated and irradiated groups during the first 6 days of tumour growth Thereafter a clear difference can be seen, the body weight in irradiated groups being less than in untreated animals

TABLE 1

The Survival Time, Total PCV of Tumour Cells and Total Cell Free Fluid Volume in Male and Female Irradiated and Control Mice Injected Intraperitoneally with Ehrlich s Ascites Carcinoma (Entries means ± SD)

Groups		No of animals	Survival time (days)	PCV (ml)	Fluid (ml)
Irradiated	♂	15	10 1 ± 3 8	0 92 ± 0 54	1 68 ± 1 23
Irradiated	♀	15	14 0 ± 3 6	0 87 ± 0 64	1 61 ± 1 50
Control	♂	13§	14 4 ± 1 9	3 08 ± 1 45	7 91 ± 4 41
Control	♀	14*	14 9 ± 2 8	2 78 ± 1 27	8 11 ± 3 79

* 1 lost due to technical error § 2 lost due to technical error

Survival time The mean values with standard deviation are given in Table 1 In the male groups the survival time was significantly reduced in irradiated mice as compared to control mice ($P<0\ 005$) There was no difference in the female groups The sex difference was significant in

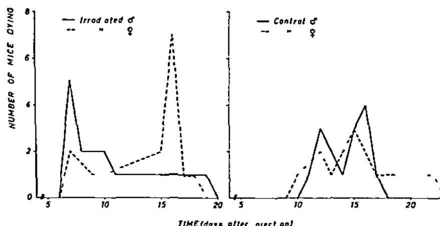


Fig 2

The distribution of deaths of irradiated and control male and female mice after intraperitoneal injection of Ehrlich's ascites carcinoma

the irradiated mice ($P<0.005$) but not in the controls. The distribution of deaths related to the survival time is shown in Fig 2. In both control groups and in the irradiated females the curves indicate two peaks, while only one peak is present in the irradiated males. The first peak comes early in both irradiated groups compared to controls, and it is most marked in irradiated males.

Total PCV of tumour cells and cell free fluid volume. The mean values with standard deviation are shown in Table 1. Neither in the irradiated nor in the control groups did the PCV of the tumour cells and the fluid volume show any relationship to survival time. Thus both sets of data could be analysed by comparing mean values. Both the amount of tumour and fluid volume were significantly reduced in the irradiated groups ($P < 0.005$). No sex differences appeared within the irradiated or control groups.

TABLE 2

Regression of the Total PCV of Erythrocytes (y) on Survival Time (x) in Male and Female Irradiated and Control Mice Injected Intraperitoneally with Ehrlich's Ascites Carcinoma.

Groups		No of animals	Coefficient of correlation (r)	Equation of regression line ($y = a + bx$)	t_b	P ($b = 0$)
Irradiated	♂	15	− 0.6624	$y = 26.4 - 1.6x$	3.188	< 0.01
Irradiated	♀	15	− 0.6963	$y = 23.7 - 1.3x$	3.935	< 0.01
Control	♂	13§	− 0.2201	$y = 14.5 - 0.5x$	0.802	> 0.40
Control	♀	14*	− 0.5098	$y = 21.1 - 1.0x$	2.053	> 0.05

* 1 lost due to technical error   § 2 lost due to technical error

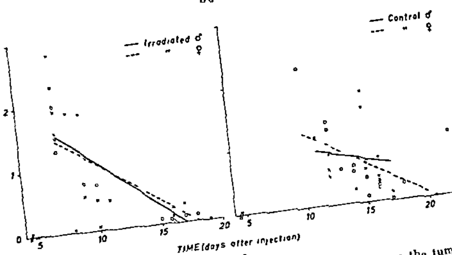


Fig 3

The scatter diagram and regression lines for the relationship between the tumour blood content and the survival time in irradiated and control male (X) and female (O) mice after intraperitoneal injection of Ehrlich's ascites carcinoma

Total PCV of erythrocytes The relationship between the total blood content and the survival time was investigated and the results are demonstrated in Table 2 This shows that there is a significant negative correlation between blood content and survival time both in irradiated males ($P < 0\ 01$) and females ($P < 0\ 01$) The correlation is still negative but *not* significantly so in the control groups Thus the mean values of the blood content can not be directly compared The scatter diagram *and* the regression lines are shown in *Fig* 3 Comparing the slopes of the lines no *significant* differences were demonstrable between the experimental and control groups (analysis of regression coefficients) On inspection of Fig 3 the levels of the regression lines within each group can be seen to be similar However, it appears from the scatter diagram that such irradiated males as represent the early and initial peak in the survival curve have a high blood content in their tumours

Solid tumour growth Some infiltration of the tissues was seen Its degree was similar in all groups

In the control group of 10 mice given irradiation *only* there was no mortality, no weight loss, and *no* clinical signs of illness

DISCUSSION

It has previously been shown that the mice of the closed colony kept at this Institute fall into two groups as regards their survival time following the intraperitoneal injection of EAC (*Hartveit* 1961) mice in one group die soon after transplantation, their tumours containing more blood than tumours of animals which die later

In the present experiment the survival curves indicate two peaks in

the control groups as demonstrated by *Hartveit*. In the irradiated males and females this bimodal distribution has changed, as the initial peaks come earlier than in the controls. While this has been of no consequence to the overall survival of the irradiated females, the irradiated males show a particularly marked first peak, no second peak and consequently, a decrease in overall survival time. The first peak in the survival curve has been closely related to increased tumour blood content (*Hartveit* 1961), and as seen from the survival curves and the scatter diagram in the present experiment this relationship is still there in all groups. However, an increased tendency to early bleeding is seen both in irradiated males and females. It is most marked in the irradiated males and the reduced survival time in this group may be explained by this increase in bleeding tendency which probably is an irradiation effect. The decrease in survival time can hardly be due to radiation effect per se as no deaths occurred among the mice which got irradiation only.

The sex difference in survival time found in irradiated animals may be explained by a greater tolerance to radiation in female mice, as demonstrated by *Rugh & Clugston* (1955). A greater capacity in female mice to resist the consequences of bleeding can not be excluded.

Following intraperitoneal injection of EAC to normal mice, a significant increase in the total number of tumour cells and ascitic fluid with time is usually found. This increase is exponential until a certain maximum is reached (*Klein & Revesz* 1953), after which the death of the animals follows. As all data in the present experiment, both in irradiated and control groups, were obtained at time of death, this may explain the lack of relationship between tumour and fluid volume, and survival time in these mice.

The PCV of the tumour cells as well as the cell-free fluid volume were decreased in the irradiated groups. This is in accordance with the findings of *Mazurek & Duplan* (1959). According to *Wheatley & Easty* (1964) and to the data presented in this and in the previous experiment (*Thunold* In press) the difference in tumour growth in irradiated and control mice seems to appear only in later phases as it could not be demonstrated during the first week following transplantation.

The mechanism of this retardation of tumour growth in irradiated mice of both sexes is not immediately clear. An abrogated immune response did not affect the tumour amount significantly on the 7th transplantation day (*Thunold* In press), and it is thus unlikely that this response has been of great importance in the present experiment either, as antibody production can be found in mice as early as 4 days after antigen injection (*Gorer et al* 1959). On the other hand as pointed out by *Spärck* (1962) and *Wheatley & Easty* (1964) it can be argued that immune reactions in normal mice may stimulate the inflammatory response and thus aid tumour growth.

The findings presented in the previous experiment, however, suggest

that anoxic tumour cell damage due to an increased bleeding tendency may play a part in the inhibition of tumour growth A depressing effect of irradiation on inflammatory reactions and serous exudation has been demonstrated in virus infections (*Rowe* 1956) and it is possible that sufficient exudate formation is also of importance in the growth of ascitic tumours. It thus seems reasonable to suggest that the difference in tumour amounts in irradiated and control mice may be as dependant on the radiation effect on inflammatory reactions and exudate formation as on its capacity to produce a haemorrhagic diathesis

As tumour growth was considerably retarded in irradiated mice, and, as also demonstrated by *Wheatley & Easty* (1964), as these animals did not survive the untreated hosts, the cause of the late deaths in irradiated groups deserves further notice No deaths occurred among the mice which got irradiation only and deaths, due to bacteriaemia of enteric origin, which may occur in mice after a single exposure to X-rays (*Miller et al* 1951), are therefore unlikely Proteolytic enzymes (see *Abercombie & Ambrose* 1962) and a polypeptide, toxic to normal cells in tissue culture (*Holmberg* 1962), have been demonstrated in the tumour ascites and serum of tumour bearing mice. These substances, produced during tumour growth, may be responsible in part for the death of mice with ascitic tumours In mice irradiated prior to tumour inoculation the tumour cells show considerable anoxic damage (*Thunold* In press) and substances toxic to the host organism may be released in greater amounts Thus inhibition of tumour growth may be accompanied by intoxication of the host in which case no increase in survival time could be expected

SUMMARY

The survival time and the growth of Ehrlich's ascites carcinoma has been investigated in normal and irradiated male and female mice

The survival time was found to have decreased in irradiated male mice, but not in the irradiated females

The irradiated animals of both sexes showed early intraperitoneal bleeding, probably as a result of irradiation damage This bleeding tendency was most marked in the irradiated male mice and it was considered the cause of the decreased survival time in this group The change in immune response in the irradiated groups, has apparently no direct influence on survival time

The amounts of tumour and of cell-free exudate were decreased in irradiated mice investigated at time of death and no sex differences were demonstrable

The consequences of host reactions to intraperitoneal tumour volume are discussed It is concluded that the decrease in tumour development in irradiated animals probably is due partly to the inhibitory effect of irradiation on inflammatory reactions and partly to anoxic tumour cell damage as a result of an increased bleeding tendency

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OBSERVATIONS ON VISNA VIRUS INFECTED CELL CULTURES STAINED WITH ACRIDINE ORANGE

By

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Received 8 x 65

In previous publications visna virus has been described as medium sized spherical particles formed by budding at the cytoplasmic membrane of the host cells and surrounded by an envelope covered with projections about 10 mμ in length (*Thormar* 1961, *Thormar & Cruickshank* 1965) In its morphology and mode of formation visna virus is reminiscent of myxoviruses and might therefore be assumed to be RNA containing On the other hand, a study of the effect of 5-bromodeoxyuridine and actinomycin D on the multiplication of visna virus has indicated that its reproduction is dependent on formation and function of DNA (*Thormar* 1965) Since staining with acridine orange is a reliable method for distinguishing between RNA and DNA within cells and for detection of abnormalities in the concentration or location of nucleic acids due to virus infection (*Armstrong* 1956, *Armstrong & Niven* 1957, *Anderson, Armstrong & Niven* 1959) this method was applied to visna virus infected cell cultures in an attempt to obtain information about any detectable alterations in the distribution or in the amount of nucleic acids occurring in the cells at various times after infection

MATERIALS AND METHODS

Cell cultures—Like in all previous studies with visna virus serially propagated cultures of sheep cells derived from the choroid plexus (*Sigurdsson et al* 1960) were employed The fibroblast like cells were grown in medium 199 containing 20 per cent sheep serum and maintained in medium 199 with 2 per cent sheep serum The monolayer cultures used for staining with acridine orange were grown on 11 × 22 mm coverslips in Leighton tubes

Infection with virus—Confluent cell layers were infected with a large amount of visna virus strain K 796 10th tissue culture passage The Leighton tubes were then rotated in a roller drum at 37° C until the coverslips were removed for staining and examination with the fluorescence microscope Sometimes the excess seed virus was removed by washing of the cell layers after an adsorption period of 2-4 hours

Staining technique—The cell layers were washed briefly with phosphate buffered saline pH 7 1, and fixed in Carnoy s fluid for 5 10 minutes at room temperature

After fixation they were dehydrated in graded d... with McIlvaine s citric acid d sodium phosphate buffer ... and stained with 0 01 per cent acrid ne orange ... buffer for 5 10 minutes After a quick rinsing wi... the coverslips were mounted in fresh buffer on sta...

D gest on with nucleases—Nucleases (Sigma C... specificity of the staining with acrid ne orange ... mal and infected cell layers were incubated either in a ... lized deoxyribonuclease (DNase) in 0 022 M ... 0 003 M $MgSO_4$ or in b) 0 05 per cent 5 X crystall... Ilvaine s buffer pH 4 0 Incubation was at 37° C f... [illegible]

film was used for colour photography

RESULTS

Examination of cells stained at various times after ... unstained cell layers did not show any cytopath... after infection a number of multinucleated and st... served after fixation and staining with acridine ... of free virus in the fluid was $10^{3.1}$ $TCID_{50}$ per ml ... nuclei looked normal with large red fluoresc... green fluorescing chromatin The red fluorescence ... stellate cells was markedly increased as compared ... (Fig 1a and b)

In cultures examined 2 and 3 days after infect... ber of affected cells was greatly increased but ... neration yet The stellate cells looked rather ... more greenish yellow nuclei on top of each ... tained large red fluorescing nucleoli and the ... matin seemed in most cases to be normal How... appeared shrunken with increased yellow flu... fluoresced uniformly orange-red and separat... material could usually not be seen The ... escence was markedly increased in stellate ... red processes stretching out from the cytop... cells without cytoplasmic processes show... fluorescence than the stellate cells The t... $TCID_{50}$ per ml in the 2 day cultures and ... tures

Four days after infection many cells ... and detach from the glass surface Ther... the nuclei The chromatin was accumula... with brilliant yellow fluorescence These ... the gla s surface after the complete d... nucle i could be seen The cytoplasm

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Fig 1

Monolayer cultures of sheep cells stained with acridine orange

a Normal cell culture
b Cell culture stained one day after infection with visna virus
c Multinuclear stellate cell stained 3 days after infection









cells was covered with blebs which like the whole cytoplasm fluoresced intensely orange red. The titre of free virus in the fluid was $10^{3.1}$ $TCID_{50}$ per ml.

Greenish yellow fluorescence could not be detected in the cytoplasm of infected cells at any time and in the nuclei red fluorescence was confined to the nucleoli.

Effect of treatment with nucleases.—Greenish yellow fluorescence did not appear in the nuclei of normal or infected cells when these were incubated with DNase before staining with acridine orange. The red fluorescence of the nucleoli remained unchanged as well as that of the cytoplasm.

After treatment with RNase orange red fluorescence did not appear in the cells by staining with acridine orange. The nuclei fluoresced greenish yellow and the cytoplasm showed a faint greenish fluorescence which was somewhat more intense in the stellate cells than in normal cells. This greenish fluorescence was not reduced by pretreatment of the cells with both RNase and DNase.

DISCUSSION

When comparing normal and infected cell cultures stained with acridine orange red fluorescence was found to be markedly more intense in the latter. The increase in fluorescence was mainly observed in the cytoplasm of multinuclear stellate cells which usually fluoresced brilliantly orange red. The intensity of the red fluorescence might partly be due to increased thickness of the stellate cells indicated by the fact that the nuclei were often found in two or more layers on top of each other. However, stellate cells which did not appear thick and showed clearly distinguishable nuclei also showed an increased red fluorescence. It therefore seems likely that this was to a certain extent caused by an increase in the concentration of stained material. Since the red fluorescence did not appear after digestion with RNase it can be concluded that it was caused by staining of RNA. The present study therefore indicates that there is an increase in the RNA content of the cytoplasm of cells infected with visna virus.

The nuclei of infected cells looked remarkably normal until the end of the infection when the cells began to degenerate. No change could be seen in the amount or location of nuclear DNA in cells showing pronounced CPE of visna virus, and DNA could not be detected in the cytoplasm.

Thus, the present observations are in accord with previous findings indicating that visna virus is an RNA virus. The DNA which seems to be synthesized early in the latent period and to be required for formation of visna virus (*Thormar* 1965) is apparently not detectable by the staining method and is not likely to play a role as bulk material for production of virus.

SUMMARY

The cytoplasm of cells infected with visna virus showed an intense red fluorescence after staining with acridine orange apparently due to increased content of RNA Until the cells began to degenerate, the nuclei looked normal with intact nucleoli and normally distributed chromatin Increase in greenish-yellow fluorescence could not be de tected

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ENZYMATIC ACTIVITIES OF *TOXOPLASMA GONDII*

By

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Received 27 1 66

In the search of a metabolic deficiency which might account for the obligatory intracellular parasitism of *Toxoplasm gondii* the presence of different enzymes involved in primary energy transformation and in addition, the occurrence in the parasites of certain enzymes with a lytic function have been studied

Different histochemical methods were employed and quantitative determinations of oxygen consumption were carried out manometric ally

MATERIAL AND METHODS

90 per cent of the mouse cells present in the exudate The remaining cells were avoided in the tests as described in the following The final parasite suspensions contained about one million parasites per ml As suspending medium Hanks solu tion was used

Histochemical methods Cytochrome oxidase was assayed according to *Burstone* (4) using 8 amino 1,2 3 4 tetrahydroquinoline as the coupling agent After incuba tion postchelation was performed in 10 per cent cobaltous acetate formalin solution The specificity of the staining reaction was studied by adding potassium cyanide to a final concentration of 10^{-3} M

Tests for lactate glucose 6 phosphate and fructose 1 6 diphosphate dehydrogenases were carried out according to *Hess et al* (10) by linking nicotinamide adenine dinucleotide (NAD) or nicotinamide adenine dinucleotide phosphate (NADP) and using Nitro Blue tetrazolium as final electron acceptor

Succinic dehydrogenase activity was assayed as described by *Nachlas et al* (19) using Nitro Blue tetrazolium as electron acceptor The control tests were carried out by omitting the substrates

Tests for diaphorases were carried out according to the method of *Pearse (21)* using $NADH_2$ and $NADPH_2$ as substrates Acid phosphatase activity was tested on unfixed as well as on formalin fixed parasites according to *Burstone* (3) using naphthol AS TR phosphate as substrate and pararosaniline as coupling agent and according to *Barka & Anlersson* (1) using α naphthyl acid phosphate as substrate and pararosaniline as coupling agent

Unfixed and also formalin fixed parasites were assayed for peptidase activity using l leucyl-α naphthyl amide as substrate according to *Nachlas et al* (20)

This study was supported by a PHS research grant AI 05074-03 from the National Institute of Allergy and Infectious Diseases Public Health Service U S A and grant N 560 from the Sw[illegible] M[illegible]al R[illegible]earch C[illegible]uncil

The skilful techn[illegible] a[illegible] s *Inger Bohwall* is gratefully acknowledged

Measurements of oxygen uptake The oxygen uptake was measured manometrically in Cartesian divers according to the method of *Zeuthen* (22) with a few minor modifications The manometers had the same internal diameter as the ones used by *Hyden et al* (11) The capillaries used for the ampulla diver were drawn from Pyrex tubing and had an outer diameter of around 0 3 mm The divers weighed about 0 5 mg their charge was about 0 2 µl and the seal consisted of a mixture of bees wax and resin with a melting point of 57° C According to *Chakravarty & Zeuthen* (6) the braking effect of the divers was checked before use The divers selected were those into which water was sucked by capillary forces at a speed of 12 15 mm/min because divers with narrower capillaries were found very difficult to handle during measurements

The flotation medium used was 0 0379 M phosphate buffer in isotonic saline The pH was adjusted to 7 4 although this would mean that around 5 per cent of the CO remained unabsorbed The error thus introduced was considered of minor importance compared to the advantage of not causing accidental damage to the parasites by alkaline fluid seeping along the diver wall into the diver charge The average rate of oxygen uptake was found to be the same at pH 7 4 as at pH 9 0 due to the considerable variation between the individual measurements within a series Each experiment was carried out using 6 manometers for the experimental material and 2 manometers for diver blanks

The divers were heat sterilized and the buffer autoclaved prior to use The medium in the flotation vessels was changed every day

The parasite suspensions were purified in the cold and Hanks solution was used as the suspending medium The divers were allowed to equilibrate in the flotation vessels for one hour before the readings were started The divers were made to float at a pressure close to atmospheric The blanks were stable within $\pm 0\ 6 \times 10^{5}$ µl/hour except for a few experiments which were ignored

RESULTS

Histochemical Observations

Activity of all the assayed oxidative enzymes was demonstrated in *Toxoplasma gondii* Cytochrome oxidase activity was observed in the cytoplasm *localized in 3 7 usually 5 small distinct granules There* was no intranuclear activity Cyanide inhibited the reaction

Succinic dehydrogenase activity was only demonstrated at a low intensity The dye precipitate for this enzyme as well as for lactate glucose 1 phosphate and fructose 1 6 disphosphate dehydrogenases was restricted to a few cytoplasmic granules

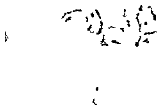


Fig 1

Toxoplasma gondii *stained for demonstration of acid phosphatases at pH 5*
The sites of activity are indicated in the cytoplasm by red staining
(naphthol AS TR and pararosaniline)

Diaphorase activity was demonstrated as distinct darkly stained granules in the cytoplasm No nuclear structures were stained In the controls without the substrate no darkly stained granules were observed

Activity of acid phosphatase as well as of leucyl aminopeptidase was demonstrated in the cytoplasm One or two prominent dye spots usually one at each end of the parasites were observed as well as small granules and a diffuse cytoplasmic staining The most intensive staining were observed for acid phosphatases (Fig 1)

Oxygen Consumption

The divers were charged with purified parasite suspension under a dissecting microscope care being taken that no blood cells entered the divers The parasites could not be seen properly much less counted once they were inside the capillary The number of parasites had to be estimated either indirectly by the volume of the diver charge and the concentration of parasites in the suspension or by counting the parasites of fixed and stained preparations made of the diver charge after the respiration measurements The latter procedure gave at the same time a good control of the purity of the parasite suspension in the individual divers

In order to make preparations for counting the parasites of the divers it was necessary to break off one end of the ampulla diver to remove the parasites Consequently the dry weight of the diver could not be determined and therefore the change in pressure read on the manometer during the experiment could not be transferred into actual consumption of oxygen It remained thus either to count the parasites and obtain a change in gas volume only in terms of change in pressure or to determine the oxygen consumption for an approximate volume of the parasite suspension

Divers could be selected in such a way that their weight volume and charge varied less than 10 per cent The errors involved in determinations of oxygen consumption by means of the ampulla divers amount at least to 10 per cent when different divers are compared Consequently errors within this range in determinations of parasite number volume weight etc should not influence the results

Some series of experiments were carried out in which the final step was counts of parasites in others the divers were weighed and the results were pooled In this way 500 1000 parasites were found to be a suitable diver charge and the oxygen consumption at 37° C was around 0 5 × 10^{6} µl/hour and parasite Single determinations within one series of 6 divers may vary with a factor of 2 above and below this estimated average value This corresponds to the scatter in results obtained with other materials (22) In different series of experiments calf serum or human serum in concentrations varying up to 20 per

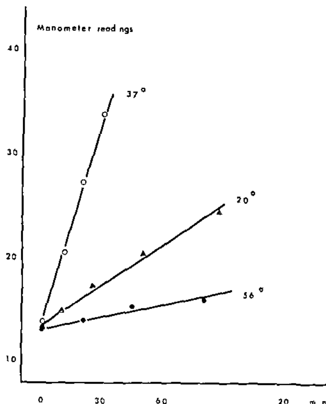


Fig 2
The relative rate of oxygen uptake of suspensions of Toxoplasma gondii *at different temperatures*

cent were added to the parasite suspension with no apparent effect on the respiration

In two series of experiments the effect on the rate of respiration of the incubation temperature was studied Aliquots of a parasite suspension were kept at 20° C 37° C and 56° C respectively for 30 min and the rate of oxygen uptake was then measured at 37° C As seen in Fig 2 where the average values of the two series of experiments are shown a rise in temperature from 20° C to 37° C increased the respiratory activity, whereas a previous incubation at 56° C damaged the parasites and caused a rate of oxygen consumption which hardly differed from that of the blanks

Effect of Enzyme Inhibitors on the Oxygen Uptake

Experiments with cyanide and adenosine triphosphate (ATP) The effect of cyanide was studied by adding the compound to an aliquot of purified suspension of *Toxoplasma gondii* After one hour at room temperature three divers were charged with the suspension and the oxygen uptake was measured at least during a two hour period In the

same experiment three divers were charged with the untreated suspension Two divers served as blanks In another series of experiments two divers were charged with parasites to which cyanide had been added, two divers with suspension containing cyanide and ATP, and two divers with untreated aliquots of the parasite suspensions

TABLE 1

Effect of Cyanide and ATP on the Oxygen Uptake of Toxoplasma Gondii *at 37° C and pH 7 4*

Molarity of KCN	ATP Molarity of	Oxygen uptake*
		100
	10^{3}	86 ± 20
10^{3}		0 7 ± 0 4
10^{3}	10^{3}	0 0
10^{4}	–	34 ± 16
10^{4}	10^{3}	82 ± 26

* Expressed in percentage of the value for untreated parasites with the standard errors indicated

TABLE 2

Effect of Fluoride and Magnesium Ion on the Oxygen Uptake of Toxoplasma Gondii *Suspended in Magnesium Free Hanks' Solution at 37° C and pH 7 4*

Molarity of NaF	Molarity of $MgSO_4$	Oxygen uptake*
	Hanks solution	110 ± 15
–		100
10^{2}	–	33 ± 12
10^{1}	–	8 ± 5
10^{1}	10^{1}	48 ± 11

* Expressed in percentage of the value for untreated parasites, with the standard errors indicated

In Table 1 the average results from 3 experiments using cyanide alone or in combination with ATP are given The oxygen uptake had a constant rate during the measurements The oxygen consumption is expressed in per cent of the value determined for the untreated parasites ATP reduced the respiration, and used in a millimolar concentration it did not influence the almost total inhibition exerted by the millimolar concentration of cyanide However the reduction to one third of the respiration caused by 10^{-4} M of cyanide was not found if ATP was also added

Experiments with fluoride and Mg Using the same technique as described above, the effect of adding sodium fluoride to a parasite suspension with or without the further addition of magnesium sulphate was tested The magnesium sulphate had been omitted in the

parasite-suspending medium This omission did not seem to influence the rate of the oxygen uptake The rate of oxygen uptake was expressed in per cent of the average value found for parasites suspended in magnesium-free Hanks' solution

In Table 2 the average values found for 3 experiments and for each concentration and combination are given A fluoride concentration of 10^{-2} M reduced the oxygen uptake to around one-third, and a concentration of 10^{-1} M gave a reduction to about one-tenth This effect could, in part, be counteracted by adding 10^{-1} M Mg^{++}

Contrary to the results obtained with fluoride no inhibition was found using 10^{-2} M of 2,4 dinitrophenol The average percentage from 10 series of experiments was 88 ± 16

DISCUSSION

Using the Warburg technique *Fulton & Spooner* (7) found an average oxygen consumption of 1.2×10^{-7} µl per parasite and hour On the average 4.9×10^{8} parasites from a pure preparation of parasites were employed for each determination They found a maximum in the rate of respiration at pH 7.4 (8)

In the present work a pronounced temperature dependence in the rate of oxygen consumption was demonstrated The rate of oxygen consumption at 37° C, pH 7.4 was found to be around 0.5×10^{-6} µl per parasite and hour, using around 500–1000 parasites per determination The difference between this rate and the one reported by *Fulton & Spooner* may be caused by differences in the methods employed for the preparation of parasite suspensions The technique used in the present work seems to be more gentle but separated less efficiently the parasites from the contaminating mouse cells In the preparation of parasite suspensions *Fulton & Spooner* have used buffered saline as the suspending medium Respiration of parasites kept in buffered saline without sugar is less than respiration of parasites suspended in media containing glucose (7)

Technically the ampulla diver method is very exacting, but requires only about one millionth of the number of parasites necessary for the determinations using the Warburg flasks It cannot be excluded that the diver charge in the present study occasionally contained single mouse cells In fact, a few were occasionally found, when preparations of the diver charge were examined after the measurements However, this should not invalidate the results obtained since a single leucocyte is known to respire at a rate of around 10^{-6} µl O_2/hour (13), and we found an oxygen uptake per diver in the order of at least 2×10^{-4} µl hour

The morphology of *Toxoplasma gondii* seen with the electron microscope has been summarized by Ludvik (14) who in a schematic representation of the parasite indicated the occurrence of 4 mitochondria In histochemical studies *Capella & Kaufman* (5) using tetrazolium

stains found that glycolytic dehydrogenases, dehydrogenases of the Kreb's cycle and diaphorases were demonstrable, localized in discrete deposits, 6 to 8 in number By staining with Janus Green B these foci were identified as mitochondria In the present study the activity of the mitochondrial enzymes studied were also found to be strictly localized to foci in the cytoplasm in a way that corresponds well with the localization of the mitochondria observed in the morphological studies

Fulton & Spooner (8) demonstrated bands of cytochrome a, b and c in the parasites In the present study activity of cytochrome oxidase was demonstrated histochemically In conformity with findings by *Fulton & Spooner* respiration was almost totally inhibited by cyanide, showing that the action of oxidases is responsible for most of the respiration That the inhibition by CO reported (8) was less complete, may mean that other oxidases than cytochrome oxidase are active in the parasites In the work of *Barnett* (2) it was reported that ATP was capable of reversing the mitotic block produced by cyanide or anaero biosis In the present report it was found that the cyanide inhibition of the respiratory activity may be counteracted by addition of ATP

Several dehydrogenases are inhibited by monoiodoacetate, which blocks the –SH groups Therefore the inhibition of the parasite respiration by this compound (8) yields little specific information but the presence of succinic dehydrogenase and malonic dehydrogenase was demonstrated histochemically (5), and the reported inhibition by arsenite (8) indicates the action of oxidative decarboxylation

It seems that glucose may be utilized by the parasite through aerobic glycolysis Thus hexokinase has been demonstrated in parasite homo genates (8) and enolase activity was indicated by the inhibition of the oxygen uptake by fluoride The fluoride forms a magnesium fluorophosphate complex and thus removes Mg^{++} from the enzyme An accumulation of the precursors of phosphopyrovate should thus occur That the fluoride in fact is inhibiting the oxygen uptake by binding the Mg^{++} necessary for the enolase activity is supported by the finding that addition of Mg^{++} prevents the inhibition, at least in part

The accumulated information strongly indicates that the mitochondrial enzymes are fully represented in *Toxoplasma gondii* Consequently, as poined out by *Capella & Kaufman* (5) "the metabolic requirements which make this organism unable to survive extracellularly are probably not directly associated with energy production" (5)

In the present report the presence of lysosomal enzymes was demonstrated histochemically The occurrence of such enzymes in the parasites may explain the prolongation of the survival time of extracellularly located parasites by the addition of, *e g* serum proteins (12, 15, 16) Perhaps the proteins supply necessary nutrition, which may be utilized in the metabolism of the parasite

In ultrastructural studies of the morphology of *Toxoplasma* no lysosomes have been described, but the localization in the cytoplasm of

lytic enzymes is not incompatible with the assumption, that the toxonemes or the paired organelle (14) may have a lysosomal function in the parasite It is of interest that some mucolytic enzymes have a promoting effect on the penetration of *Toxoplasma* into cultured host cells (17) Furthermore, preparations of lysed parasites exert such a penetration-promoting activity (18) It seems thus possible that lysosomal enzymes may contribute to the parasites' ability to penetrate the walls of the host cells

SUMMARY

By means of histochemical studies and the use of ampulla diver respirometry the presence of mitochondrial enzymes in *Toxoplasma gondii* was studied The respiration was found to be about 0.5×10^{-6} µl/O_2 per hour and parasite at 37° C and pH 7 4 No deficiency in the oxidative or glycolytic enzyme activity was demonstrable It is concluded that a possible metabolic deficiency of the parasite impelling the necessity of a host cell for the parasite reproduction could not be correlated to an insufficiency in respiratory enzymes Proteolytic enzymes were demonstrated histochemically and a hypothesis concerning a possible lysosomal activity of the toxonemes is presented

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COUNTER-CURRENT DISTRIBUTION OF POLIOVIRUS TYPE 2 AND 3[1]

By

STELLAN BENGTSSON

Received 1 iii 66

The study of marker characteristics of poliovirus strains has become increasingly important with the use of avirulent live vaccines and especially in the analysis of vaccination incidents Among the most widely used markers are the rct marker for ability to multiply at temperatures above 37° (*Lwoff* 1959, *Carp & Koprowski* 1962), the *d* marker for ability to form plaques under acid overlay (*Vogt et al* 1957, *Hsiung & Melnick* 1958), the *m* marker for sensitivity to sulphated polysaccharides (*Nomura & Takemori* 1960, *Takemoto & Liebhaber* 1962), the *e* marker for avidity to the anionexchanger DEAE-cellulose (*Boeyé* 1963), and the serodifferentiation marker (*Mc Bride* 1959, *Wecker* 1960), which expresses the relation between the strain studied and other strains within the same type, this latter marker characteristic has been extensively used for the study of isolates from cases connected with vaccination incidents

Of the markers mentioned the *d*, *e* and *m* markers are correlated to each other as shown by *Agol & Chumakova* (1962) and *Bengtsson et al* (1964), and it has been suggested that they are due to differences in the composition in the protein coat of the virus although this difference is not great enough to give different isoelectric points *m* and *m* mutants are known with many different viruses *e g* encephalomyocarditis virus (*Liebhaber & Takemoto* 1963) foot-and mouth disease virus (*Dinter & Sibalin* 1958) herpes simplex virus (*Takemoto & Fabisch* 1964) and myxoviruses such as influenza A and B (*Takemoto & Fabisch* 1963) It has been shown by amino acid analysis that the *m* and *m* mutants of encephalomyocarditis virus differ in the protein composition (*Moscarello & Kaighn* 1964) and similar studies on various mutants of poliovirus are in progress

It has been reported earlier that differences between strains of poliovirus type 1 can be demonstrated by counter-current distribution in aqueous polymer phase systems of dextran sulphate and polyethy-

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1 This investigation was supported by grants from the *Damon Runyon Memorial Found* the *Jane Coffin Childs Memorial Fund* the *Swedish Medical Research Council* (B 66 33) and the *Medical Faculty of Uppsala University*

lene glycol (*Bengtsson & Philipson* 1963) The distribution pattern is correlated to the *m* marker *m* strains having a higher affinity for the dextran sulphate rich bottom phase than *m* ones The counter current distribution may also be used to eliminate m^+ virus from an *m* virus pool

This paper describes the behaviour of several strains of poliovirus type 2 and 3 in the polymer phase system earlier used for the study of type 1 and also demonstrates that counter current distribution may be a useful tool for the isolation of *m* mutants from *m* virus populations

MATERIAL AND METHODS

Viruses The strains investigated are listed in Table 1 They were usually tested after one passage in primary monkey kidney cultures in this laboratory

Cell Cultures

Monolayer cultures of cynomolgus kidneys were prepared according to *Youngner* (1954) and grown in bottles and plastic petri dishes in Eagle s minimal essential medium (MEM) (*Eagle* 1959) with 6 per cent calf serum After outgrowth the cultures were changed to Eagle s MEM without serum and used for plaque assays and production of virus A calf serum adapted strain of HeLa cells originally adapted to horse serum (*Mandel* 1961) was grown in Hank s solution enforced with 0 1 per cent lactalbumin hydrolysate and 0 1 per cent yeast extract and containing 20 per cent calf serum In some experiments a continuous monkey kidney cell line BSC-1 kindly provided by *Dr Hans Dinlerholm* of the Department of Virology was used It was grown in Eagle s medium with 10 per cent calf serum

Virus Assays

Virus was diluted appropriately in phosphate buffered saline (PBS) and inoculated in 0 1 ml amounts onto cultures in plastic petri dishes previously washed with PBS Adsorbtion was carried out for 30 minutes at 37° and was enhanced by placing the plates on a test tube rocker specially adapted for culture plates Four ml of agar overlay was then added It contained 0 05 per cent lactalbumin hydrolysate 0 225 per cent sodium bicarbonate 2 per cent calf serum and 0 95 per cent Special agar Noble (Difco) in Earle s salt solution for the monkey kidney cultures and 0 175 per cent sodium bicarbonate 4 per cent calf serum and 0 95 per cent agar in MEM for the BSC-1 and HeLa cells

Marker Test

Tests for the *m* marker were carried out by plating virus under overlay containing 0 01 per cent dextran sulphate 2000 (DS) generously supplied by AB Pharmacia Uppsala Sweden A difference in titre of normal size plaques of more than 2 log between plates with and without dextran sulphate was required for a strain to be regarded as *m*

Counter Current Distribution

This was carried out with an automatic fractionator (F C Apparatus Company 538 Walnut Lane Swarthmore Pennsylvania) using batteries containing 20 tubes The system used for the fractionation was the same as had been used in the studies of poliovirus strains of type 1 and contained 7 per cent (w/w) sodium dextran sulphate 500 (limiting viscosity 0 50 dl g kindly supplied by AB Pharmacia Uppsala Sweden) 1.2 per cent (w/w) polyethylene glycol (PEG Carbowax Carbide and Carbon Chemical Co New York) in 0 6 M sodium chloride buffered to pH 7 0 with 0 01 M sodium phosphate buffer The phase system was prepared in batches by dissolving the appropriate amounts of chemicals in deionized water the system which does not form phases at room temperature was then placed in the cold (+ 4° C for 48 hours when the impurities present had collected at the inter

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face between the phases and at the bottom of the vessel used Equal amounts of top and bottom phase were then sucked off mixed and kept in the cold until used for experiments When the batteries were loaded the phase system was placed at 37° C until the phases had disappeared and then thoroughly mixed by stirring

The batteries which were adapted to 18 ml of system were filled by adding phase system to 19 of the 20 tubes by means of a Cornwall syringe The last tube was then filled with system containing the virus to be studied This was made up by mixing 10 5 g DS 500 20 per cent 3 6 g PFG 6 000 10 per cent 4 5 g 4 M CaCl 3 g 0 1 M phosphate buffer pH 7 2 1 g virus suspension and water to 30 g Counter current fractionation was then carried out at +4° C. The mixing time was 7 minutes separating time 30 minutes and 19 transfers were carried out in each experiment

After completion of the fractionation the batteries were placed at 37° C until phases had disappeared when the tubes were emptied One ml of the fractions were then added to 1 ml 1 M KCl in order to precipitate the sodium dextran sulphate which would otherwise interfere with the infectivity assay for *m* variants The precipitate was spun down and the supernate assayed for infectivity by the plaque procedure described above

The approximate partition coefficient K, *i e* the concentration of virus in the top phase/concentration of virus in the bottom phase was calculated according to the following formula $K = \frac{r_{max} + 0\ 5}{n + 0\ 5 - r_{max}}$ where r_{max} designates the number of the tube containing the maximum amount of virus and n is the number of transfers r_{max} is calculated according to the formula $r_{max} = \frac{0\ a + 1\ b + 2\ c + \quad + n\ z}{a + b + c + \quad + z}$ where 0 1 2 n designates the number of the tube and a b c x is the amount of virus found in each tube

The recovery of infectious virus after the counter current analysis was usually about 100 per cent and only rarely did it fall below 70 per cent

RESULTS

Type 2 Strains

m character and counter current distribution The type 2 strains listed in Table 1 were tested for their *m* character and analysed for their counter current distribution pattern The *m* type 1 strain LSc 2 ab was included as a control As seen in Table 2 which also gives the approximate partition coefficients for these strains all strains except the Wistar strain are *m* according to the criteria used The results of the counter current distribution experiments which are given in Fig 1, show that the m^{+} strains MEF₁ and P712, Ch 2 ab distribute in favour of the top phase with peaks in fraction 14 15 whereas the *m* Wistar strain had its peak in fraction 5 The result with the *m* strain 9327 was similar to that obtained with strains MEF₁ and P712 Ch 2 ab

The distribution pattern for the Wenner strain however, revealed heterogeneity of its population with one peak in fraction 3 and one in fraction 16 (Fig 2 A) Samples from these two peaks were treated with equal amounts of 1M KCl and were then passaged once in monkey kidney cell cultures The resulting virus harvests were subsequently examined for their *m* character and counter current distribution pattern The results which are given in Fig 2 B and C Table 3 show that the progeny from fraction 3 distributes in favour of the bottom phase with a peak in fraction 1 and 2 and is *m* , whereas the progeny of fraction 16 which is *m* has its peak in fraction 14 to 16

TABLE 2

m Character and Partition Coefficient of Type 2 Strains

Strain	Titre in log PFU per 0.1 ml: normal overlay	Titre in log PFU per 0.1 ml: overlay containing 0.01 % DS[1]	m character	Partition coefficient K
P 712, Ch, 2 ab	7.0	6.9	+	3.2
Wenner Lot 55	7.3	7.0	+	heterogeneous
Wistar	6.7	4.1	—	0.43
MFF$_1$	7.3	6.8	+	2.4
9323	6.8	7.1	+	3.1
L Sc 2 ab (type 1 control)	7.1	4.0	—	0.3

[1] DS dextran sulphate 2000

TABLE 3

m Character of the Progenies of Fraction 3 and 16 after Counter Current Distribution of the Wenner Strain of Poliovirus type 2

Strain and fraction	Titre in log PFU per 0.1 ml: normal overlay	Titre in log PFU per 0.1 ml: overlay containing 0.01 % DS	m character	Partition coefficient K
Wenner lot 55	7.3	7.0	+	heterogeneous
Fraction 3	7.0	4.9	—	0.39
Fraction 16	7.3	7.3	+	2.4

Isolation of m mutants from an m^+ population It has been shown earlier (*Bengtsson & Philipson* 1963) that the *m* type 1 strain LSc 2 ab contains m^+ mutants and that these mutants may be isolated by means of counter-current distribution Since it has also been shown that *m* mutants may arise from m^+ populations (*Takemori & Nomura* 1960) it was investigated whether counter-current distribution could reveal the presence of such *m* mutants in an m^+ virus population

The Sabin strain P 712, Ch, 2 ab was fractionated by counter-current distribution and material from fraction 0 was alternately passaged in monkey kidney and fractionated by counter-current distribution

After 2 cycles of counter current distribution and passage the pattern had changed from that characteristic of the m^+ strains into the *m* pattern with the peak in fraction 4 as shown in Fig 3

The same material was also analysed for *m* character and a difference in 2.3 log was found between the titre under normal overlay and that under overlay containing 0.01 per cent dextran sulphate

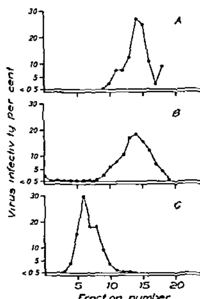


Fig 1

Counter-current distribution pattern of three strains of poliovirus type 2 *A* P712 Ch 2 ab, *B* MEF$_1$, *C* Wistar Infectivity is given as the percentage of the recovered virus found in each fraction.

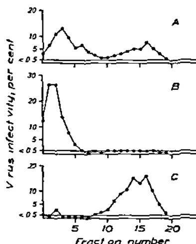


Fig 2

Distribution pattern of the type 2 strain Wenner and its derivatives *A* Original strain, *B* Progeny of fraction 3, *C* Progeny of fraction 16

Correlation between plaque size and effect of dextran sulphate on virus multiplication When the effect of dextran sulphate on the plaque size of the *m* strain Wistar and the *m* mutant from strain P 712, Ch 2 ab was compared it was found that the Wistar strain was clearly

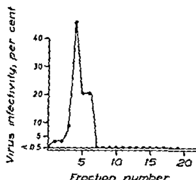


Fig 3
Distribution pattern of the *m* mutant of the type P 712 Ch, 2 ab

more inhibited This is shown in Fig 4, which also shows the plaques of the m^+ strain P 712, Ch, 2 ab for comparison

It was also studied whether any differences could be demonstrated in multiplication experiments These were carried out as multistep growth experiments to enhance the effect of dextran sulphate, which acts only on the adsorption of *m* mutants of poliovirus type 1 to sensitive cells (*Bengtsson* 1965)

Approximately 100 PFU of the three strains mentioned above were adsorbed to six plates of HeLa cells for 30 minutes at 37° C Three plates for each strain were then supplied with 5 ml of Eagles MEM and three plates with the same medium containing 0 05 per cent of dextran sulphate 2000 The plates were then incubated and one plate per strain and medium withdrawn after 24, 32 and 40 hours They were frozen at —60°, thawed, and assayed for infectivity by plaque titration in HeLa cells The results, which are given in Fig 5, show a correlation between plaque size and the inhibitory effect of dextran sulphate The multiplication of the Wistar strain, which forms small plaques under overlay containing dextran sulphate is more inhibited than that of the *m* variant of P 712 Ch, 2 ab whose plaques under dextran sulphate overlays are not so conspicuously reduced The multiplication of the m^+ strain P 712, Ch, 2 ab, the plaque size of which is not reduced by dextran sulphate, is not inhibited by the presence of dextran sulphate in the concentration used Similar results were obtained when the experiment was carried out in BSC-1 monkey kidney cells

Type 3 Strains

m character and counter current distribution The sixteen strains of poliovirus type 3 listed in Table 1 were examined for *m* character and subjected to counter-current distribution As shown in Table 4 all strains were m^+ according to the criteria used and all strains distributed in favour of the top phase with partition coefficients between 4 6 and 6 6 The distribution pattern of some of the strains is shown in Fig 6

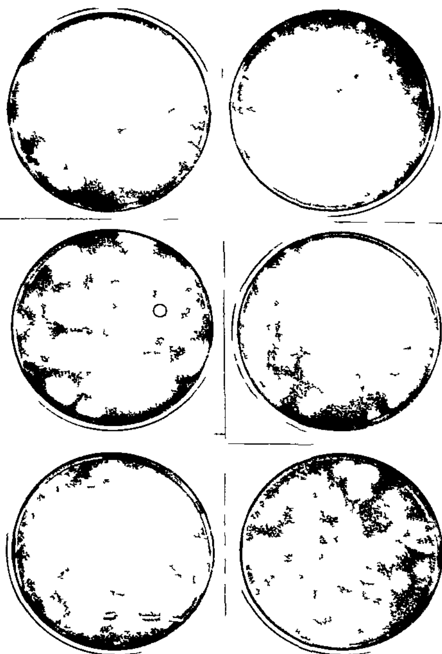


Fig 4

Plaque morphology of t pe ? stra ns in monkey kidney cells From top to bottom the Wistar strain the *m* mutant of P 712 Ch 2 ab and P 712 Ch ? ab Left normal overlay right overlay conta ning 0 01 per cent dextran sulphate ?000

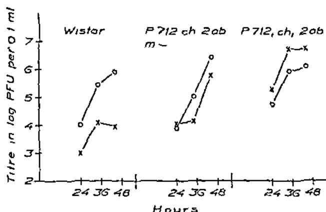


Fig 5
Multiplication of type 2 strains
0–0 in Eagles MEM, x x in Eagles MEM with 0 05 per cent dextran sulphate 2000

The results of the counter-current distribution experiments were reproducible within rather narrow limits; *e g* thee different experiments with the strain Leon 12 a₁ b gave partition coefficients of 4 8, 5 4 and 5 9 corresponding to r_{max} values of 16 1, 16 4 and 16 6, respectively

Attempts to isolate m mutants The isolation of an m^- mutant of the type 3 strain Saukett after passage in HeLa cells has been described (*Takemori & Nomura* 1960) The strains P 040, Saukett and Leon 12 a₁ b

TABLE 4
m Character and Partition Coefficient of Type 3 Strains

Strain	Titre in log PFU per 0 1 ml normal overlay	Titre in log PFU per 0 1 ml overlay containing 0 01 %	*m* character	Partition coefficient K
Leon 12 a_1 b	7 3	7 0	+	5 4
P 001	6 6	6 4	+	5 9
P 040	6 7	6 6	+	6 6
2 F	7 2	7 2	+	5 5
3 F	6 8	6 8	+	5 1
255 W	7 6	7 2	+	4 6
P 24	6 8	6 7	+	5 8
Wistar *M III*	7 5	7 3	+	4 9
Wenner	7 5	7 5	+	5 8
Lederle	6 5	6 3	+	5 0
Glenn	6 8	6 5	+	4 7
Saukett	7 0	7 3	+	5 8
9894	6 9	6 8	+	4 8
USOL D bac	6 6	6 4	+	5 5
USOL E bec	6 1	6 4	+	4 6
Saukett baa	6 4	6 7	+	4 7
LSc 2 ab (type 1 control)	7 1	4 0	—	0 3

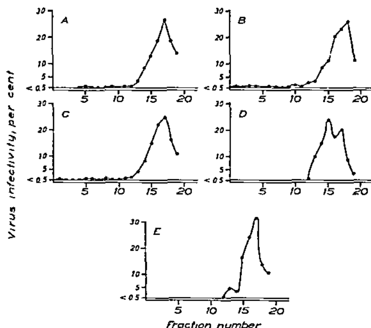


Fig 6

Distribution pattern of five strains of poliovirus type 3 *A* Saukett, *B* Leon 12 a_1 b, *C* Wistar M III, *D* Lederle, *E* *P* 24

were rapidly passaged in primary monkey kidney and HeLa cell cultures This was carried out by exposing washed plate cultures to 0 1 ml of undiluted virus material for 30 minutes at 37° after which they were supplied with 5 ml of Eagles MEM The cultures were frozen as soon as they showed cytopathic effects, thawn and new cultures inoculated with 0 1 ml of the harvested material Tests for *m* character were carried out after each passage After ten passages the strains were still m^+ and counter-current distribution analysis showed that they had retained their original distribution pattern

Since it had been found that an *m* mutant could be isolated from the m^+ type 2 strain P 712, Ch, 2 ab by means of alternating counter-current distribution and culture passage, the strain Leon 12 a_1 b was treated in the same way After counter-current distribution a sample from fraction 0 was treated with 1 M KCl in order to remove the dextran sulphate and 0 1 ml inoculated onto primary monkey kidney cell cultures in the manner described above The virus harvest was then fractionated After eight cycles of this treatment no change had occurred in the distribution pattern and the viral progeny from fraction 0 was found to be m^+

DISCUSSION

The results of this work confirms the earlier findings with poliovirus type 1 as regards the correlation between *m* character and counter-current distribution pattern (*Bengtsson & Philipson* 1963) It is thus demonstrated that *m* strains, *i e* strains that are inhibited by dextran sulphate distribute in favour of the dextran sulphate rich bottom phase in counter-current distribution whereas m^+ strains, *i e* strains that are unaffected by dextran sulphate, *do not* show any *preference* for the dextran sulphate phase It has been shown elsewhere (*Bengtsson et al* 1964, *Bengtsson* 1965) that this is mainly due to absorption of the virus by the polyanion The reason for this difference in affinity to poly anions is at present unknown but could be due to difference in the amino acid composition in the viral protein coat Such differences have been reported for *m* and m^+ variants of encephalomyocarditis virus (*Moscarello & Vaughn* 1964)

It has been demonstrated by *Agol & Chumakova* (1962) that the *d* marker, *i e* the inhibition of plaque formation of sensitive strains at a low pH, is most probably due to the presence of sulphated polysaccharides in the agar overlay It is thus to be expected that the *d* and *m* marker which consequently are both due to the action of sulphated polysaccharides should show covariation It is therefore noteworthy that several of the virus strains examined in this study *e g* the strains P 712, Ch, 2 ab and Leon 12 a₁ b, (*Vogt et al* 1957), USOL-D bac USOL-E bec and Saukett baa (*Vonka et al* 1964) and Glenn (*Barnes et al* 1963), have been reported to be *d* although they show m^+ character and distribute as m^+ strains in counter-current distribution analysis

The reason for this apparent discrepancy between the *d* and *m* test is unknown, but might possibly be explained by differences in the technique used in the *d* test Most *d* tests have been done in rhesus kidney cultures whereas the *m* tests in this study were performed in cynomolgus cells *Voss* (1964), however, studied the effect of dextran sulphate on the plaque size of the vaccine strains of Sabin in rhesus cultures and found the type 2 and 3 strains to be unaffected by 0 01 per cent dextran sulphate 2000 The reason for the difference must thus at present be left unexplained

The *d* and *m* character is usually associated with avirulence and it has been shown (*Takemoto & Kirschstein* 1964) that an m^+ mutant from the *m* vaccine strain LSc 2 ab produced histological lesions in high proportion in monkeys after intrathalamic inoculations It is also generally agreed that the live vaccine strains of poliovirus type 3 are those that are most liable to give rise to "vaccination incidents (*Furesz et al* 1964 *Henderson et al* 1964) It is therefore noteworthy that none of the attenuated type 3 strains tested was *m* and that no *m* mutant could be isolated by the use of methods that had proved effective for the isolation of an *m* mutant from the *m* type 2 strain P 712, Ch, 2 ab

It was found by comparison of the partition coefficients for the *m* strains of poliovirus type 2 and 3 that the type 2 strains had K values between 2 4 and 3 5 whereas the K values of the type 3 strains varied between 4 6 and 6 6 The reason for this difference is not known but is probably explained by differences in the composition of the viral protein coat which are to be expected with virus strains of different antigenic types

SUMMARY

Counter-current distribution analysis of five strains of poliovirus type 2 and sixteen strains of poliovirus type 3 showed a correlation between the distribution pattern and the *m* character tested as the ability to form plaques under overlay containing 0 01 per cent dextran sulphate An *m* mutant of type 2 could be isolated from an m^+ strain by means of alternating counter current distribution and cell culture passages No *m* strain of type 3 was found and attempts to isolate *m* mutants by methods used with the *m* type 2 strain failed

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THE BACTERIOSTATIC ACTIVITY OF SULPHONAMIDES IN THE PRESENCE AND ABSENCE OF PROTEIN

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The main advantages of the new long-acting sulphonamides in the treatment of infectious diseases are their slow excretion and their ability to maintain high blood concentrations with fewer and lower dosages than those previously employed in sulphonamide therapy The high blood concentrations so easily obtained are thought to be due partly to the high degree of protein binding that occurs with these drugs and partly to the molecular structure of the various derivatives The protein binding was at first considered a great improvement, and a number of new compounds were manufactured, all possessing a very high degree of protein-binding Some of the recently developed sulphonamide derivatives show a protein binding of almost a hundred per cent, e g sulphadimethoxine, 98 7 per cent bound, and sulphaethidole 98 7 per cent bound, both at serum concentrations of 0 4 μmol ml (22)

The advantage of this high protein binding has been questioned as concerns the bacteriostatic effect of the long acting sulphonamides Actually, the first investigations concerned with the problem of protein-binding and bacteriostatic effect of sulphonamides were published in 1942, by *Davis* (8) and *Davis & Wood* (11), that is, before the introduction of the long-acting sulphonamides (sulphaethidole and sulphamethoxypyridazine, 1956, sulphadimethoxine, 1958) These investigators, in experiments with seven sulphonamides, stated that "A quantitative correlation has been demonstrated between the protein binding tendency and the bacteriostatic activity of the 7 drugs" In his next publication *Davis* (9) stated that "It is probable that only the unbound drug is bacteriostatically active" On the introduction of the long-acting sulphonamides, these suggestions of Davis gained new importance

A survey of the available literature concerning long acting sulphonamides, discloses that the bacteriostatic ineffectivity of the protein-bound part of a sulphonamide is generally accepted This means, that

in the case of most long-acting sulphonamides, only a very small fraction of the chemically determined concentration in blood is considered active This view is based on *in vitro* experiments A more thorough study of the literature reveals, however, that publications concerning the bacteriostatic effect of sulphonamides almost exclusively refer to only two publications of recent date treating the problem of protein-binding and bacteriostatic activity

Newbould & Kilpatrick (21) published experiments where the effect of human serum and bovine albumin on the bacteriostatic activity of several sulphonamides against a strain of *Staphylococcus aureus* had been investigated They stated that protein bound sulphonamide has no antibacterial activity

Anton (3) found in experiments very similar to those of Newbould and Kilpatrick that albumin bound sulphonamide was not available for antibacterial action This was, however, found to be valid only at very low sulphonamide concentrations When the concentrations were increased to about 10 mg%, ' The effect of the binding was found to be much reduced"

Apart from these investigations, most studies on sulphonamides have been concerned with the determination of the extent of the protein binding, and with *in vivo* experiments as a measure of the effectivity of the various compounds The results obtained from *in vivo* experiments with highly protein bound sulphonamides do not agree with the findings discussed above from *in vitro* experiments Many reports have shown that highly protein bound sulphonamides have a very good effect when tested *in vivo* (13, 16, 17) Clinical experience with highly protein bound sulphonamides has also been reported to be very satisfactory (7, 12)

Several theories have been put forward to explain this discrepancy *Davis* (10) stated that the protein bound sulphonamide is to be considered as a reservoir, and that the sulphonamide will be available for bacteriostatic action as a result of a dissociation of the protein—sulphonamide complex Recently *Bunger* (5, 6) also found that the protein-bound part of a sulphonamide is bacteriostatically inactive, but the bound molecules are set free again, and are, accordingly, not lost for bacteriostatic action This view is now shared by most sulphonamide investigators (12, 14)

Bünger (5) stresses, however, that only the concentration of unbound sulphonamide in plasma and extracellular fluid ('Gewebswasser") *is of real importance*

Since the results obtained in experiments with sulphonamides *in vivo* and *in vitro* do not agree very well, and also as a limited number of *in vitro* experiments concerned with the bacteriostatic activity of sulphonamides in the presence of protein have been *published*, it was decided to carry out some bacteriostatic experiments on *these lines* The first group of experiments has been published earlier (18), and has

been heavily criticized (4 25) as high dilutions of the sulphonamide containing sera were employed in the bacteriostatic titrations

Naturally, dilution of the sera with protein free media will shift the existing *equilibrium between protein bound and unbound sulphonamide* a higher percentage of unbound sulphonamide being present in the dilutions For this reason our experiments have been continued and the present paper gives the results obtained when testing the bacteriostatic activity of two sulphonamides in the presence of higher protein concentrations

The sulphonamides investigated were sulphadimethoxine (4 sulphanilamido 2 6 dimethoxypyrimidine) and sulphadiazine (2 sulphanilamidopyrimidine) Sulphadiazine was chosen because no sulphonamide has yet been found that is a better bacteriostatic agent and also because its protein binding is comparatively low (45 1 per cent bound at serum concentration 10 0 mg% (22)) It is recognized as the standard sulphonamide to which all others are compared Sulphadimethoxine on the other hand is known to be one of the most highly protein bound sulphonamides available (98 7 per cent bound at serum concentration 12 4 mg% (22)) If any difference in the bacteriostatic activity of sulphonamides in the presence of protein could be demonstrated it was thought likely that it would occur with these compounds

As previously (18) the dilution technique in liquid medium was chosen This technique is generally considered to be a practical and reproducible method for the determination of bacteriostatic activity of chemotherapeutics (20) A strain of *Shigella dysenteriae* was the test organism employed in the first experiments described This organism had previously been found to be highly sensitive to sulphonamides when tested in protein free medium Later experiments were performed with two strains of *Escherichia coli*

METHODS

The bacteriostatic activity of sulphadimethoxine and sulphadiazine was tested in the presence of varying amounts of human serum and in some experiments in the presence of bovine albumin[1] Control titrations were performed in protein free solutions of the sulphonamides Sterile sera from healthy humans were used Only sera showing normal total serum protein values and normal electrophoresis patterns were employed The sera were kept at −20° C in small portions and a fresh portion was used for each experiment

For the albumin solution 3 5 g bovine albumin was dissolved in sterile buffered sodium chloride solution (see below) to give 100 ml

Stock standard solutions of the sulphonamides[2] 400 mg in 100 ml were also prepared in buffered sodium chloride solution (0 9 per cent sodium chloride in distilled water was adjusted to pH 7 4 with 0 1 molar Sørensen s phosphate buffer) For further use the sulphonamide solutions were diluted with the medium to be

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[1] *Armour Pharmaceutical Company Ltd England*

[2] The sulphonamide preparations used were the following Sulphadimethoxine Madribon obtained from F Hoffmann LaRoche & Co A G Basel sulphadiazine obtained from A/S Synthetic Denmark

used for the bacteriostatic titrations This medium was the semisynthetic medium described by *Adams & Roe* (2) which is known to be free of sulphonamide inhibitors

The test organism, *Shigella dysenteriae III* was stored on Dorset's egg medium For inoculation, an 18 20 hours culture on Adams' and Roe's medium was used The two strains of *Escherichia coli* were stored on ordinary nutrient agar, and 18–20 hours cultures were prepared as for the *Shigella* strain The size of the inoculum will be given for each experiment in the respective tables Each test tube was inoculated with one small drop of the suitably diluted inoculum, and the tubes were incubated at 37° C for 18 hours The titres are given as the last dilution of sulphonamide showing no growth

In the first experiments three standard series were prepared for each sulphonamide The sulphonamide solutions were diluted with medium to give the following concentrations of sulphonamide in a total volume of 1 05 ml 0 20, 0 10 0 09 0 08 0 07, 0 06 0 05 0 04 0 03, 0 02 and 0 01 mg% (i e mg/100 ml) One ml of each dilution was pipetted into three series of test tubes In the first series 0 05 ml of bovine albumin solution was added to each tube, in the second series 0 05 ml of inactivated human serum was added to each tube, and in the third series, 0 05 ml of the protein free medium was added to each tube Each tube was inoculated with 200 300 organisms of the *Shigella dysenteriae* and the experiment was repeated several times as will be shown in the tables (Tables 1 and 2) Control tubes containing no sulphonamide were prepared for each series in each experiment After incubation, these were diluted serially (10 fold) with 0 9 per cent sodium chloride solution, and 0 1 ml of each dilution was transferred to Petri dishes containing ordinary nutrient agar After incubation for 24 hours at 37° C the organisms were counted

For further investigations with the addition of larger amounts of human serum to the sulphonamide solutions, it was necessary to use a heavier inoculum in order to obtain good growth with this organism Titrations were carried out with the addition of 10 per cent and 20 per cent inactivated serum The number of organisms per tube was approximately 1 7 million With this inoculum size the growth was equal in controls containing only medium and those with medium containing 20 per cent serum The final sulphonamide concentrations used in these series were 20 0 10 0 5 0 2 5 1 25 0 625, 0 313, 0 156, 0 078, 0 039 0 019 and 0 009 mg% in a total volume of 1 ml in each test tube

In further experiments, two strains of *E coli* were used as test organisms The strains employed were chosen as they had been found to show a relatively high sensitivity to sulphonamides in protein free medium and good growth in high serum concentrations The bacteriostatic activity of sulphadimethoxine and sulphadiazine was tested against both these organisms in the presence of 25 per cent and 50 per cent inactivated human serum The sulphonamide concentrations used in the titrations were as follows 12 0, 11 0 10 0 8 0 5 0 4 0 3 0 2 0 1 5 1 0 0 5 and 0 25 mg% Each test tube was inoculated with approximately 2500 organisms When a more diluted inoculum was used these organisms did not grow well in as much as 50 per cent serum With the present dilution the control tubes showed that similar growth occurred in all three series for each strain i e in protein free medium, medium with 25 per cent serum and medium with 50 per cent serum The incubation time and reading of results was as before

RESULTS

Tables 1 and 2 show the results obtained in the bacteriostatic experiments where bovine albumin and human serum had been added to solutions of sulphadimethoxine and sulphadiazine, and a small inoculum of the test organism *Shigella dysenteriae* had been used Counting the organisms in control tubes from each series showed equal growth in all tubes The addition of bovine albumin to sulphadimethoxine resulted in a slight increase in the minimum inhibitory concentration from 0 06 to 0 078 mg% When serum was added to the sul-

phadimethoxine solutions, the minimum inhibitory concentration was almost doubled, from 0.06 to 0.11 mg%. Neither the addition of bovine albumin nor human serum produced any changes in the bacteriostatic activity of sulphadiazine.

TABLE 1

Bacteriostatic Effect of Sulphadimethoxine against Shigella dysenteriae *III in the Presence of Protein*

Medium	Minimum inhibitory concentration mg% Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Arithmetic mean
1.00 ml protein free medium + 0.05 ml bovine albumin solution*	0.07	0.08	0.09	0.09	0.06	0.08	0.078
1.00 ml protein free medium + 0.05 ml human serum	0.09	0.09	0.10	0.10	0.08	0.20	0.110
Standard 1.05 ml protein free medium	0.07	0.05	0.06	0.06	0.06	0.06	0.060

* 3.5 g bovine albumin/100 ml. Inoculum size: 200–300 organisms.

TABLE 2

Bacteriostatic Effect of Sulphadiazine against Shigella dysenteriae *III in the Presence of Protein*

Medium	Minimum inhibitory concentration mg% Exp. 1	Exp. 2	Exp. 3	Exp. 4	Exp. 5	Exp. 6	Arithmetic mean
1.00 ml protein free medium + 0.05 ml bovine albumin solution*	0.07	0.08	0.09	0.05	0.05	0.06	0.067
1.00 ml protein free medium + 0.05 ml human serum	0.07	0.10	0.06	0.05	0.06	0.07	0.068
Standard 1.05 ml protein free medium	0.07	0.06	0.07	0.06	0.05	0.08	0.067

** 3.5 g bovine albumin/100 ml. Inoculum size: 200–300 organisms.*

Tables 3 and 4 show the results obtained when larger amounts of human serum (resulting in 10 per cent and 20 per cent) were added to solutions of sulphadimethoxine and sulphadiazine, and these were titrated against a heavier inoculum of *Shigella dysenteriae*. The minimum inhibitory concentration for both sulphonamides when titrated with this inoculum in protein free solution was 0.078 mg%. The minimum inhibitory concentration of sulphadimethoxine was 8 times greater when the serum concentration was 10 per cent and 34 times

greater when the serum concentration was 20 per cent For sulphadiazine the minimum inhibitory concentration was doubled when the serum concentration was 10 per cent, and was 14 times greater when the serum concentration was 20 per cent

TABLE 3

Bacteriostatic Effect of Sulphadimethoxine against Shigella dysenteriae *III in the Presence of Human Serum*

Serum added to the medium	Minimum inhibitory concentration, mg%						Arithmetic mean
	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6	
No serum	0 078	0 078	0 078	0 078	0 078	0 078	0 078
10 % serum	1 250	0 625	0 625	0 313	0 625	0 625	0 677
20 % serum	2 500	2 500	5 000	2 500	2 500	1 250	2 708

Inoculum size 1 7 mill organisms

TABLE 4

Bacteriostatic Effect of Sulphadiazine against Shigella dysenteriae *III in the Presence of Human Serum*

Serum added to the medium	Minimum inhibitory concentration mg%						Arithmetic mean
	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6	
No serum	0 078	0 078	0 078	0 078	0 078	0 078	0 078
10 % serum	–	0 156	0 156	0 156	0 156	0 156	0 156
20 % serum	2 500	1 250	1 250	0 625	0 625	0 313	1 094

Inoculum size 1 7 *mill organisms*

TABLE 5

Bacteriostatic Effect of Sulphadimethoxine against E coli strain 2 in the Presence of Human Serum

Medium	Minimum inhibitory concentration of sulfadimethoxine mg%						Arithmetic mean
	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6	
Protein free	1 5	1 5	1 5				1 5
25 % serum	8 0	8 0	5 0				7 0
Protein free	1 5	1 5	1 5	1 5	1 5	1 5	1 5
50 % serum	8 0	8 0	8 0	8 0	8 0	12 0	8 7

Inoculum size 2 500 organisms

Table 5 shows the minimum inhibitory concentrations of sulphadimethoxine in the presence of 25 per cent and 50 per cent serum when titrated against *E coli*, strain '2' Table 6 gives the results obtained when *E coli*, strain "11" was the test organism The latter seems to be

a little more resistant to this sulphonamide, but the difference is small. The minimum inhibitory concentration of sulphadimethoxine was about 5 times greater both when the serum concentration was 25 per cent and when it was 50 per cent.

Tables 7 and 8 give the corresponding results obtained with sulphadiazine. For sulphadiazine solutions the minimum inhibitory concentration was 2–3 times greater when serum was added. The difference between the effect of 25 per cent and 50 per cent serum was small.

TABLE 6

Bacteriostatic Effect of Sulphadimethoxine against E. coli *strain 11 in the Presence of Human Serum*

Medium	Minimum inhibitory concentration of sulphadimethoxine mg%					Arithmetic mean
	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	
Protein free	2.0	1.0	2.0			1.7
25 % serum	8.0	8.0	8.0			8.0
Protein free	2.0	1.5	2.0	4.0	1.5	2.2
50 % serum	12.0	8.0	12.0	11.0	8.0	10.2

Inoculum size 2 500 organisms

TABLE 7

Bacteriostatic Effect of Sulphadiazine against E. coli *strain 2 in the Presence of Human Serum*

Medium	Minimum inhibitory concentration of sulphadiazine mg%							Arithmetic mean
	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	Exp 6	Exp 7	
Protein free	1.0	1.0	1.0					1.0
25 % serum	1.5	1.5	1.5					1.5
Protein free	0.5	1.0	1.5	2.0	1.0	1.0	1.5	1.2
50 % serum	4.0	1.5	1.5	4.0	4.0	4.0	2.0	3.0

Inoculum size 2 500 organisms

TABLE 8

Bacteriostatic Effect of Sulphadiazine against E. coli *strain 11 in the Presence of Human Serum*

Medium	Minimum inhibitory concentration of sulphadiazine mg%					Arithmetic mean
	Exp 1	Exp 2	Exp 3	Exp 4	Exp 5	
Protein free	1.0	1.0	1.0			1.0
25 % serum	2.0	2.0	2.0			2.0
Protein free	1.0	1.0	1.0	1.0	1.0	1.0
50 % serum	3.0	3.0	4.0	5.0	2.0	3.4

Inoculum size 2 500 organisms

DISCUSSION

All the techniques available for determination of the bacteriostatic activity of chemotherapeutics have their limitations It seems, however, that if care is taken, the dilution method in fluid medium has fewer drawbacks than most other methods The important points to watch are the medium, the inoculum size and the dilutions employed for the titrations

The medium must be free of sulphonamide inhibitors, such as peptone and agar The semi-synthetic medium described by *Adams & Roe* (2) fulfils this requirement, and most organisms will grow well on it It is also comparatively easy to prepare without any changes from batch to batch

The inoculum size should be as small as possible, and the dilutions employed in the titrations should preferably have shorter intervals than the serial twofold dilutions usually employed in bacteriological titrations When these conditions are fulfilled, it is not difficult to obtain good reproducibility when titrating sulphonamides in protein-free solutions The difficulties arise when serum is added in fairly high concentrations, as most sulphonamide sensitive organisms do not grow well when too much serum is added to the medium Accordingly, one has to use a larger inoculum in order to obtain good growth A compromise has to be made, and for this reason the present experiments have been performed with not more than 50 per cent serum in the sulphonamide dilutions The first experiments carried out with the addition of bovine albumin and human serum to sulphonamide solutions (Tables 1 and 2) showed that bovine albumin and human serum did not have the same effect on the antibacterial activity of sulphadimethoxine Therefore if investigations of this kind are to be performed, one should use human serum when comparison is to be made with conditions *in vivo* This point is frequently neglected It was evident that the addition of human serum in the concentrations employed in these experiments did reduce the antibacterial activity of sulphadimethoxine but not that of sulphadiazine against this test organism (*Shigella dysenteriae*) Experiments were therefore continued with the addition of larger amounts of serum

The results obtained when 10 per cent and 20 per cent human serum were added to solutions of sulphadimethoxine and sulphadiazine (Tables 3 and 4), left no doubt as to the inhibitory effect of human serum on the antibacterial activity of these sulphonamides Comparing the increase observed in the minimum inhibitory concentration of both sulphadimethoxine and sulphadiazine with the figures given by *Rieder* (22) for the protein-binding of these sulphonamides, 1 3 per cent and 54 5 per cent unbound sulphonamide, respectively, in undiluted serum it can be seen that the increase in the minimum inhibitory concentration of sulphadiazine (14 times) is considerably higher than might be

expected The increase in the minimum inhibitory concentration of sulphadimethoxine (34 times) was found to be comparatively lower. The results obtained in experiments with *E coli* as test organism, and the addition of 25 per cent and 50 per cent human serum, were also unexpected In these experiments the minimum inhibitory concentration of sulphadimethoxine was found to be 5–6 times higher in 25 per cent and in 50 per cent serum than in protein-free solution, and that of sulphadiazine was found to be 2–3 times higher than in protein-free solution The latter value seems reasonable compared with the protein binding figures but the value found for sulphadimethoxine is low in comparison For both sulphonamides the increase in the minimum inhibitory concentration in the presence of protein was much smaller when tested against *E coli*, than when the test organism was a strain of *Shigella dysenteriae* There seems to be little correlation between the protein binding figures and the antibacterial effect of these two sulphonamides when tested in as much as 50 per cent human serum In all experiments where the minimum inhibitory concentrations of sulphadimethoxine and sulphadiazine were determined in the presence of large amounts of serum, the increase in the minimum inhibitory concentration found for sulphadimethoxine was greater than that found for sulphadiazine The difference between the antibacterial activity of sulphadimethoxine and that of sulphadiazine in the presence of serum was, however, in all experiments found to be too small to correspond to the difference in the extent (per cent) of the protein-binding of these two sulphonamides The dilutions used in these titrations were too low to account for the lack of correlation

According to these results, the decrease observed in the antibacterial activity of these sulphonamides in the presence of human serum could be due to some inhibitory effect of serum itself which partly destroys the effect of the sulphonamides without this effect necessarily being *connected with the protein-binding of the sulphonamide This is in* agreement with *Kerp* (15), who, in investigations on the protein binding of penicillin, found that albumin-linked penicillin was less inactivated than expected, indicating that protein-binding is not the full answer to the loss of activity of a drug in the presence of proteins

Another explanation, which seems not unlikely, is that the strength of the protein-binding is of importance The use of this expression in the sense used by *Rieder* (22, 23) and called by him "F P B" value, "Festigkeit der Protein Bindung", also called "Ablösbarkeit", "Separability", has been attacked by *Berlin & Kruger-Thiemer* (4) as meaning less According to these authors, the term "Festigkeit der Protein-Bindung" has later been said by *Rieder* (23) to describe nothing that is not already defined by the use of Langmuir's adsorbtion isotherms the constants α and β, and the percentage of protein-bound sulphonamide This may be so Nevertheless he has published the results obtained in the experiments (23), and the conclusion was made that in-

vestigation of the separability of the plasma protein bound sulphonamides showed that for all the preparations tested, this bond is so weak and easily reversible that it suffices to dilute the plasma with an aqueous salt solution in order to dissolve the bond

Recently, similar views have been expressed by others investigating the protein-binding of sulphonamides and antibiotics *Schollan* (26) wrote "Die Starke der Bindung (defined by him by means of the binding constant k, according to the law of mass action) sagt jedoch nichts daruber aus, durch welche Mechanismen, unter welche Umständen (z B durch Verdrängungsreaktionen) und mit welcher Leichtigkeit *das Arzneimittel vom Eiweiss wieder abgelost werden kann*" The expression "Leichtigkeit" seems to express the same as Rieder's "Ablos barkeit" or "Separability", and our strength of binding There is, undoubtedly, some confusion as to the use of these expressions, and perhaps the best would be to use in English the word separability used by Rieder As this is not generally accepted, we shall continue to use the phrase strength of binding, as the opposite of the expression "Loose binding" being used by others

The belief that the *protein binding of chemotherapeutics* can *not* be described by means of the constants derived from the law of mass action only, has recently been supported by other investigators *Acred et al* (1) investigated the degree (per cent bound) and nature of the protein-binding of three penicillins, and found that penicillin derivatives showing a high degree of protein-binding may be "Loosely bound", and vice versa They stated "The *in vivo* activity, however, may not be significantly influenced if the binding is of such a loose character that the penicillin is readily available for antibacterial activity"

Mossner et al (19) investigated the protein-binding of demethylchlortetracyclin, and found that the binding was considerable about 64 per cent, but it could not be particularly strong, "A mostly loose protein binding, resulting in more effective antibiotic concentrations than could be expected with firm protein-binding"

Ruiz-Torres & Meinig (24) have studied the protein binding of sulphonamides in dialysis experiments They use Langmuir's constants α and β as measures for affinity and capacity In addition to these *constants, they characterize the protein binding of sulphonamides by* a third factor "Bindungsfestigkeit", or "Reversibility" They have come to the conclusion that the sulphonamide-binding to albumin is also a process dependent on time and, hence, affinity has to be considered as a time function The molecular weights did not appear to play a significant rôle as concerns binding capacity or albumin affinity, but with high molecular weights a greater binding strength was observed "However, two sulphonamides with identical molecular weights can have differing binding strength, suggesting that more factors are operative here"

In similar dialysis experiments performed in our laboratory (un

published experiments) we have found that there is a difference in the strength of the protein binding of the various sulphonamides as determined by continuous dialysis and this strength does not always follow the degree (extent) of the protein binding It is obvious that until more is known of the nature of the protein binding of chemotherapeutics especially that of sulphonamides, the influence of this binding on the antibacterial effect needs further investigation

SUMMARY

The bacteriostatic activity of sulphadimethoxine and sulphadiazine was tested in protein free standard solutions, and in the presence of various amounts of normal human serum An extremely sulphonamide sensitive strain of *Shigella dysenteriae* and two less sulphonamide sensitive strains of *Escherichia coli* were used as test organisms The results obtained were compared to the degree of protein binding (per cent protein bound sulphonamide) of these sulphonamides The difference between the minimum inhibitory concentration of sulphadimethoxine and that of sulphadiazine was with the test organisms employed found to be considerably smaller in all experiments than the difference between unbound sulphadimethoxine and unbound sulphadiazine

The influence of the protein binding on the antibacterial effect of the sulphonamides deserves further investigation

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VACCINIA VIRUS HI ANTIBODY BLOCKED BY A NON HAEMAGGLUTINATING EXTRACT OF VACCINIA INFECTED CAM

By

KJELD KR SKJORLAND

Received 1 iii 66

It is known that haemagglutinating virus preparations can be made non haemagglutinating without the loss of their capacity to combine with haemagglutination inhibiting (HI) antibody

Thus *Tyrrell & Horsfall* (8) reported in 1953 that influenza virus degraded by freezing and thawing by treatment with 5 M urea or by heating at 65° C, still combined with homologous antibody They demonstrated this by blocking of the haemagglutination inhibition and neutralization reactions

In 1964 *Cleeland & Sugg* (2) demonstrated this phenomenon as they investigated the effect of trypsin on influenza virus

In the present investigation it is demonstrated that a similar blocking of the HI antibody can be obtained also with vaccinia virus

MATERIALS AND METHODS

Virus

Virus suspension was obtained by infecting chorio allantoic membranes (CAM) of 11 days old embryonated eggs with vaccinia virus The membranes were harvested on the 14th day allowed 3 ml of sterile normal saline per membrane and kept at —70° C until required

Serum

Rabbits were depilated and inoculated with vaccinia virus from calf lymph by scarification After three weeks the rabbits were hyperimmunized by three successive intravenous injections of diluted calf lymph vaccine

They were bled after one week and the sera inactivated by heat 56° C for ½ hour absorbed with red blood cells of fowl and kept stored at —20° C.

Enzymes

Crystalline trypsin of the type Trypure "Novo" 22 ?5 Anson units mg was employed in the trypsin experiments 1.5 mg of Trypsin Inhibitor "Sigma" inhibited approximately 1 0 mg of trypsin

Also Lecithinase C from Clostridium Welchii Sigma" was employed The activity was stopped by heating at 56° C for ½ hour

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CAM is capable of combining with HI antibody although it has lost its haemagglutinating property

Heating at 56 or 100° C for ½ hour did not reduce the HIB titres It was also resistant to incubation with trypsin (250 microg ml) and lecithinase (200 microg ml) respectively for 4 hours at 37° C

Treatment with 0 005 M KIO₄ at 37° C for 24 hours destroyed the HIB activity as did incubation with crude pronase (500 microg ml) for the same length of time

The antigen was applied to a 1 4 × 20 cm Serva DEAE column which had been equilibrated with 0 15 M NaCl in Na K buffer (Sorensen) pH 6 8 Stepwise elution was performed with 0 15 0 20 0 25 0 30 0 45 and 0 60 M NaCl

About 80 p c of the protein was removed by the elution with 0 15 M NaCl while practically all of the HIB material was recovered in the 0 30 M fraction

All fractions of the 0 30 M NaCl eluate was dialysed against distilled water freeze dried and resuspended in 0 15 M NaCl at 1/5 of the original volume HIB tested and chemically analysed (See table)

Some Chemical Data and Titres of the Genetron Treated Vaccinia Virus Suspension G/a s 40 and of the Material Eluted with 0.30 M NaCl from G/a s 40 when Applied on DEAE Cellulose

Substrate	Protein (Folin)	microg/ml Neutral sugar (Orcinol)	Uronic acid (Carbazole)	HA	HIB
G a s 40	4100	450	275	< 2	128
0 30 M eluate concentrated 5 times	390	62	213	< 2	128
0 30 M eluate rechromatographied	360	64	< 18	< 2	64

The uronic acid content of the 0 30 M NaCl eluate was found to be strongly elevated Rechromatography of the fraction however reduced the uronic acid content in the subsequent 0 30 M fraction below detectable level without a significant loss of HIB activity

On agar precipitation the semipurified HIB preparation (G a s 40) tested against immunized rabbitserum gave one line The same did the 0 30 M fraction and the lines were identical

Rabbits were given intravenous injections of the material Primary immunization produced no detectable HI antibody while a booster dose gave a significant rise

Preparations of vaccinia infected L cells did not yield any HIB activity nor haemagglutinin

DISCUSSION

The works of *Gessler et al* (4), *Hamparian et al* (5) and *Epstein* (3) clearly showed that viruses could be purified and separated from host cell constituents if extracted from infected tissue by homogenization in a water fluorocarbon mixture Therefore the author of the present paper regards the semi purified material obtained from the supernate as associated with the virus particle itself It is not likely that the material is obtained through degrading of the virus haemagglutinin, as the treatment of virus free vaccinia haemagglutinin with fluorocarbon *did not give any HIB activity*

Appleyard demonstrated in 1962 the presence of a serum-blocking antigen in vaccinia infected tissue and found this to be a protein The report was amplified by *Appleyard et al.* (1) in 1963 In 1965 *Warnaar et al* (9) found a similar SB antigen by alkaline treatment of vaccinia virus

Whether there is a relationship between these antigens and the HIB antigen demonstrated in the present paper remains to be examined

SUMMARY

Vaccinia virus infected chorio allantoic membranes were homogenized in a water fluorocarbon mixture Repeated treatment rendered the suspension non-haemagglutinating without destroying the capacity to combine with HI-antibody

After centrifugation at 105,000 G the HIB blocking-activity (HIB-Titre) was found in the supernate Treatment with trypsin and lecithinase did not reduce the HIB-titre of the substance It was destroyed by incubation with pronase (500 microg ml) and 0 005 M KIO_4 at 37° C for 24 hours By chromatography on DEAE cellulose the HIB active material was eluted with 0 30 M NaCl

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A URONIC AND SIALIC ACID FREE CHICK ALLANTOIC MUCOPOLYSACCHARIDE SULPHATE WHICH COMBINES WITH INFLUENZA VIRUS HI-ANTIBODY TO HOST MATERIAL

3 *A High Content in Chicken Bile, and an Inactivating Enzyme in Chicken Faeces*

By

ARILD HARBOE and GUNNAR HAUKENES

Received 10 ii 66

Previous papers have described the titration (2), purification (3) and chemical composition (4) of this haemagglutination inhibition blocking (HIB) allantoic antigen, which obviously must be present also in the haemagglutinin of influenza virus from chick allantoic fluid (1) This paper describes a search for the HIB-antigen in different organs of the chicken, and the detection in chicken faeces of an HIB-antigen inactivating factor, which shows various characteristics of an enzyme

MATERIALS AND METHODS

The *testvirus* (a red cell eluate of the influenza B strain Lee), the *antiserum* (against the influenza A_0 strain PR 8) and the performance of the *HIB test* have been described earlier (1 2)

Tissue extracts Various organs were removed from 18 days old chick embryos and minced with scissors ground in a mortar suspended in saline and extracted for one hour at room temperature After centrifugation the organ extracts were examined in the HIB test

EXPERIMENTS AND RESULTS

1 *HIB-Antigen in Different Organs from Chickens*

Extracts of the following organs of chick embryos were examined Kidney, lung, heart, liver, spleen, skin-muscle, cartilage-bone and cornea Tested were also the bile, yolk, stomach content and (embryonic) red cells Allantoic and amniotic fluids and membranes had been tested earlier and found to contain the antigen (1, 2) It was found that the bile gave high HIB-titres, even higher than the allantoic fluid The bile

The authors are indebted to Mrs *Anne Marie Lauten* and Miss *Berit Salthe* for skilled technical assistance

gave agar precipitation lines attributed to the host antigen antibody reactions (3) Aside from small amounts of HIB antigen in the liver extract and the stomach content no HIB antigen could be demonstrated in the other extracts neither did the red cells absorb HIB antibodies Extracts treated overnight at 37° C with 0 1 per cent crystalline trypsin gave the same results

Bile collected from adult fowl gave an HIB titre of about 30 000 which was about 100 times greater than that of allantoic fluid This bile showed a high content of haemagglutination inhibitors against the Lee-eluate the titre being about 3 000 The bile was pooled from 500 animals on an average one ml was obtained from each one The chemistry of the HIB antigen purified from the bile will be described in a later paper — No HIB antigen was found in ox bile

2 *A Factor in Chicken Faeces which Partly Inactivates the HIB Antigen*

Since it was found that chicken bile contained great amounts of HIB antigen faeces from chickens was tested for the presence of HIB antigen The faeces was collected within 24 hours after it had been produced and was suspended in phosphate buffered saline (pH 7 2) by adding one litre of saline to 0 5 kg of faeces The suspension was filtered through paper overnight spun and passed through Seitz filter to get it bacteriologically sterile These procedures took place at 4° C The extract was then examined and found to contain considerable amounts of HIB antigen titres being about as high as that of chick allantoic fluid The routine HA and HI controls showed that haem agglutinins were low titred and normal HA inhibitors absent so they did not interfere with the HIB test

Because of the great variety of digestive fluids and bacterial products in faeces it appeared possible that one or more enzymes capable of breaking down the HIB antigen present there might be found in the faeces extract The subsequent experiments deal with this subject

In the first experiment the faeces extract was incubated overnight at 37° C while an aliquot was kept at 4° C Control tubes with penicillin and streptomycin 5000 IU per ml of each were included It was found that incubation at 37° C had reduced the HIB titre from 240 to 10 240 being the titre of the same sample kept at 4° C for the same period The same respective titres were shown by the faeces extracts to which had been added the antibiotics No visible alterations took place in the fluids especially no turbidity or sediment formation

In the next experiments it was first established that heating over night at 56° C or in a boiling waterbath for 15 minutes did not reduce the HIB titre of the faeces extract Also an HIB-antigen preparation partly purified from chick allantoic fluid showed no titre reduction when left for 4 days at 20 37 or 56° C When the faeces extract was preheated at 56° C for 2 hours incubation overnight at 37° C did not

result in reduction of the HIB-titre. Heating at 56° C for 30 minutes did not affect the HIB-titre of a faeces extract which had lost some of its activity by incubation overnight at 37° C. These experiments indicate that the faeces extract contains a factor which partly inactivates the HIB-antigen at 37° C. The factor itself was inactivated after heating at 56° C for two hours. In later experiments it was found that incubation for several days (at 37° C) some times was necessary in order to produce a significant titre reduction.

To see whether the HIB-antigen inactivating factor was released from bacteria, a broth, a glucose broth and a partly anerobic HS broth with HIB antigen added, were inoculated with chicken faeces. The cultures were incubated overnight at 37° C and then tested for HIB activity. No titre reduction was observed, which indicate that the HIB-antigen inactivating factor was not liberated from the faecal bacteria under the experimental conditions.

The activity of the HIB-reducing factor at different pH values was also examined. Acetic acid buffer and NaOH were employed to adjust the pH at the values shown in the table. Incubation was performed at 37° C for 1, 5 and 12 days, while control tubes were kept at 4° C for the same period. The results are shown in the table. It is seen, that after one day at 37° C only two times reductions of titre were observed. After 5 days the titre had dropped to one fourth and after 12 days the reductions were still more pronounced.

Influence on HIB Activity by Incubation of Chicken Faeces Extract at 4 and 37° C at Various pH Values

pH	HIB titres after incubation for 12 days at		Ratio of HIB titre after incubation at 4° C to HIB titre after incubation at 37° C for		
	4° C	37° C	1 day	5 days	12 days
8.2	64	24	2	2.7	2.7
7.6	96	9	1.5	4.6	10.7
6.9 (original)	64	8	2	8	8
6.1	64	6	2.7	4	10.7
5.0	64	12	2	2.7	5.3
4.0	128	128	1.3	1	1

Finally it was examined whether the incubation of the faeces extract at pH 4 had resulted in destruction of the HIB antigen inactivating factor. Samples of the faeces extracts which had been acidified to pH 4 and incubated at 4° C and 37° C for 12 days, were readjusted to pH 7. Each sample was then further incubated for 9 days at 4° C and 37° C respectively and tested for HIB-activity. No significant titre difference between these samples was observed, and the titres were similar to those found in the pH 4 samples 9 days earlier. The faeces extracts thus

appeared to have lost their HIB antigen inactivating capacity after being kept at pH 4

DISCUSSION

Various organs of the chick embryo were tested for the presence of HIB antigen The bile appeared to have high contents of this antigen and moderate amounts were found in the liver No HIB antigen was extracted from the other organs except for the foetal membranes as reported earlier (1 2) Also in the bile of the adult fowl the content of HIB antigen was very high In the faeces the HIB titre was similar to that of the allantoic fluid Some destruction of the HIB antigen has possibly taken place during the passage of the bile through the intestines However the *in vitro* inactivation of the HIB antigen in faeces was fairly slow

Since the HIB antigen was not detected in other organs than the liver, the antigen is either produced in the liver or it is altered before excretion in such a way that it cannot be traced back to its origin by our serological methods It was pointed out in the former article (4) that aside from the ester sulphate groups the HIB antigen showed great resemblance chemically with blood group substances One possibility is therefore that this species specific mucopolysaccharide has been sulphated in the liver and thus changed antigenically

It is not clear whether or not all the HIB antigen in the allantoic and amniotic fluids originates from the bile Further investigations concerning this are in progress

The HIB inactivating factor found in chicken faeces extracts is most likely an enzyme The following observations support this view

1) The inactivation took place at 37° C and not at 56° C or 4° C
2) It had an optimal pH about 7 it did not take place at pH 4 and only to a limited extent at pH 8 2
3) The inactivating factor was destroyed when heated at 56° C at pH 7 and when stored at pH 4 even at 4° C

Despite several attempts the authors have found no other enzyme than the one(s) in chicken faeces capable of inactivating the antibody combining capacity of the HIB antigen

SUMMARY

Great amounts of HIB antigen were found in the bile from chick embryos and adult fowls Aside from moderate amounts in the liver and the foetal membranes no HIB antigen was found in extracts of various other chick embryo organs

The HIB antigen was also demonstrated in extracts of chicken faeces

together with an enzyme which was capable of inactivating the HIB antigen Some properties of this enzyme have been reported

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INFLUENZA VIRUS HI ANTIBODY COMBINING WITH NORMAL CHICK MATERIAL AND PRODUCED IN HENS IMMUNIZED WITH CHICK GROWN INFLUENZA VIRUS

By

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Previous investigations have shown that the haemagglutination by influenza virus grown in the chick allantoic sac is inhibited by rabbit antisera against normal chick allantoic material (1, 8). The HI antibody to host material was also found in sera from persons given influenza vaccine (3) and in sera from ferrets inoculated intranasally with influenza virus infected chick allantoic fluid (1). Recent experiments have revealed that liver and bile from embryonic as well as grown up chickens contain an antigen combining with the HI antibody to normal chick allantoic material, and that this antigen is lacking in the other organs except for the foetal membranes (4). The myxoviruses acquire this host antigen when they grow in the entodermal cells lining the allantoic cavity of the chick embryo (5). It is assumed that the antigen, which is a sulphated mucopolysaccharide (6), is synthesized in the liver and then brought to the allantoic fluid. Apparently the antigen is adsorbed to the parietal allantoic cells, which finally lend it to the myxoviruses when they are budding on the cell membrane.

In the present investigation it was examined if also *chickens* injected with influenza virus grown in the chick allantoic sac might develop HI antibody combining with uninfected chick material, and this was found to occur.

MATERIALS AND METHODS

The influenza virus strains A0/PR-8 and B Lee were employed. They were grown in the allantoic sac of White Leghorn eggs. The A strain was inoculated as infected chick allantoic fluid diluted 10^{-3} into 11 days old eggs and harvested 2 days later. The B strain was inoculated into 10 days old eggs and harvested 3 days later. Mouse grown influenza virus was obtained by infecting mice through intranasal instillation of 0.1 ml of Lee virus infected chick allantoic fluid under ether anaesthesia. Four days later the surviving animals were killed and the respiratory tract washed with saline to which had been added 1 000 IU per ml of penicillin and streptomycin. The collected washings were employed as an antigen in the HI test. The other antigens consisted of red cell eluates of chick allantoic PR 8 and Lee viruses. They were

prepared by adding one per cent of packed red cells to the infected allantoic fluids at 0° C, leaving the suspensions for ½ hour, spinning down the cells and eluting the virus into saline at 37° C for 1½–2 hours. Phosphate buffered saline (pH 7.2) was employed in all experiments.

Immune sera. Three White Leghorn hens, which were a few months old, were injected intravenously with PR–8 virus eluate, having an HA-titre of 2 000 (about twice the titre of the allantoic fluid). The chickens received altogether 4 injections each time one ml. The injections were given at 3 or 4 days intervals. Blood samples were drawn before immunization and one week after the last injection. The sera were stored at —20° C until they were tested.

In addition to these sera the investigation included chicken sera prepared at the Communicable Disease Center, Atlanta, Ga., U.S.A. by Dr. *R. Q. Robinson* and distributed by WHO. The latter sera were drawn from animals which had received simultaneously an intravenous and an intraperitoneal injection with either A2/Taiwan/1/64 or B/Singapore/3/64 infected chick allantoic fluid (12).

Haemagglutination inhibition (HI) test. Before the sera were HI tested normal inhibitors were destroyed by treatment with cholera filtrate (Philips Duphar). One volume of serum was mixed with 5 volumes of cholera filtrate and incubated over night at 37° C, subsequently inactivated at 56° C for one hour, and finally absorbed at 0° C for ½ hour with 10 per cent fowl red cells in order to remove the haemagglutinins. The HI tests as well as the other titrations were performed in the World Influenza Centre 'Perspex' plates. The antibody titres correspond to 4 haemagglutinating doses of the virus, and are the reciprocal values of the primary serum dilutions showing partial (50 *per cent*) inhibition (12).

Haemagglutination inhibition blocking (HIB) experiments. In order to test if the inhibition could be blocked by the host antigen, 0.25 ml of host antigen solution was added to each serum dilution about 5 minutes before the red cells and the virus were added. As a control saline only was added instead of the host antigen solution. Purified host antigen was used in these tests. This HIB antigen was prepared from chick allantoic fluid by means of a method previously described (6). From slaughtered chickens' bile the HIB antigen was partly purified by means of a DEAE Sephadex column. The solutions employed in the HIB tests had the HIB titre 2 000 when tested with rabbit sera (2). The bile preparation showed at lowtitred HI and was therefore treated with an equal volume of cholera filtrate before it was employed in the HIB test with chicken sera.

RESULTS

Normal chicken sera and sera from the same chickens after immunization with chick allantoic PR–8 virus were treated with cholera filtrate and examined in the HI-test with a red cell eluate of chick allantoic Lee virus, and with PR–8 virus: the results are presented in the table. As expected, the chickens developed hightitred antibodies to the homologous virus (PR–8) and the anti PR–8 titres were not reduced by addition of the host specific HIB-antigen.

The antibodies reacting with the host specific antigen could be detected with the Lee virus, because there is no virus specific cross reaction between the PR–8 and Lee strains. As seen in the table, all chickens produced HI-antibody to the viral host material, the titres being 24, 96 and 48, respectively.

In order to exclude that the inhibition recorded with chick allantoic Lee virus was due to virus specific antibody, the sera were tested with mouse grown Lee virus, because mouse grown virus is not inhibited by antisera against normal chick material (1). It was then found that the mouse grown Lee was not inhibited by the anti PR–8 chicken sera. In a control test it was found that the mouse grown Lee gave about the

same HI titre as chick allantoic Lee against anti Lee rabbit serum showing no loss of antibody sensitivity by the passage in the mouse lungs

The authors failed to demonstrate HI antibody to normal chick material in the influenza immune chicken sera received from WHO This may be due to the different immunization procedures

Haemagglutination Inhibition (HI) Titres of Sera from Chickens Repeatedly Injected Intravenously with a Red Cell Eluate of the Influenza A_0 Strain PR–8 from Chick Allantoic Fluid

Chicken No	Tested with a red cell eluate of the influenza B strain Lee from chick allantoic fluid in the presence of: Saline only	Chick allantoic HIB antigen	Tested with tracheobronchial washings from Lee virus infected mice	Tested with the PR 8 virus preparation in the presence of: Saline only	Chick allantoic HIB antigen
1 Normal serum	< 6	< 6	< 6	< 24	< 24
Immune serum	24	< 6	< 6	6000	6000
2 Normal serum	< 6	< 6	< 6	< 24	< 24
Immune serum	96	< 6	< 6	3000	3000
3 Normal serum	< 6	< 6	< 6	< 24	< 24
Immune serum	48	< 6	< 6	3000	3000

In the next experiment it was examined if the inhibition of chick allantoic Lee virus could be blocked by normal host material The result is shown in the table No inhibition was seen when the chick allantoic HIB antigen had been added in the HI test It was further examined whether the preparation of chicken bile would bind the viral host specific HI antibody demonstrated in chicken sera This was found to take place as also this preparation significantly reduced the HI titres observed

DISCUSSION

Previous investigations have shown that HI antibody reacting with normal chick allantoic material can be produced in various animals However it had not yet been examined if chickens themselves might develop such antibody The present results show that this really happens The inhibition recorded could hardly be due to normal inhibitors or to virus specific antibodies and it was blocked by normal allantoic material and bile from chickens

Of particular interest is the finding that the chickens can be immunized so that the sera contain antibody reacting with chicken bile An antibody against this particular liver antigen can therefore be pro

duced in chickens, and this may have pathological significance The authors have observed that the HI-antibody provoking capacity of the host antigen is increased when it is attached to the virus particle (1)

It is possible that chronic hepatitis in humans partly is due to an autoimmune reaction (10, 11) —Recently the recovery of a hitherto unknown myxovirus from serum and urin of patients with infectious hepatitis was reported (9) If this or another virus grows in the liver cells, it could acquire the HIB-antigen there, and the antigen would then be distributed to the antibody producing cells with the blood stream, and an autoimmune reaction might result

An experimental approach to this hypothesis seems possible, because the chemical composition of the particular antigen, a sulphated muco polysaccharide, is quite well known (7) Other antigens may, however, be involved

SUMMARY

Chickens injected intravenously with the influenza virus strain A₀/PR-8 grown in the chick allantoic sac produced antihaemagglutinins against a purified preparation of the unrelated influenza virus strain B Lee This inhibition was shown to be caused by antibody against the host material of the virus, because the inhibition was blocked when normal chick allantoic material was added Also chicken bile combined with the antibody in chicken sera, and the possibility of a relationship between this phenomenon and chronic hepatitis was discussed

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DETECTION OF ANTI-γ GLOBULIN FACTORS IN HUMAN SERA BY SIMPLE SCREENING METHODS

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Received 7 ii 66

The anti-γ globulin factors in human sera comprise a group of γ-globulins, presumably antibodies, which are very heterogeneous in their specificities (see 12, 20, 21) They can be classified as iso- or heterospecific, according to their reaction with γ-globulin of isologous or heterologous origin

The largest group is the rheumatoid factors (see 9), usually γM-globulins They are demonstrated by a variety of test systems, mostly by agglutination tests using γG globulin of various species origin attached to particulate carriers

The best known and most employed test systems are those using human (isologous) or rabbit (heterologous) γ-globulin The rheumatoid factors (7 8) including most anti-Gm factors (see 20, 21), react with the Fc fragment of γG-globulin The various anti-Gm factors detect genetic determinants of human γG-globulin Rheumatoid factors which do not detect genetic characters also exhibit different specificities Such factors have been separated by various procedures by column chromatography (11, 14, 27), by mixed agglutination (18), or by inhibition dispersion and absorption (16) Factors which primarily showed no reaction with human γ globulin but reacted strongly with rabbit γ globulin were demonstrated (16, 18, 27) These investigations were performed with sera containing a mixture of factors

In the present work a larger panel of rheumatoid sera was tested for rheumatoid factors using simple screening methods Several sera showed *monospecific reactions either with human or rabbit γ globulin*

MATERIALS AND METHODS

Sera Twelve hundred sera originating from patients at the *Haukeland Hospital* Fana and at the *Hospital for Rheumatic Diseases* Haugesund were investigated All the patients were suspected of having rheumatoid arthritis (definite probable or possible and their sera were examined by the Waaler Rose test The material partly included sera used in another study (19)

This investigation was supported in part by *US Public Health Service Research Grant 5 RO5 TW 00172 01 from the National Institutes of Health*

The incomplete anti D sera were the same as used previously (19 20) Incomplete anti-CD Ripley was kindly provided by Dr *Marion Waller* of the Department of Pathology School of Medicine Richmond, Va USA

Reference anti-Gm sera were used as previously (19)

Rabbit anti sheep erythrocyte serum was prepared as described earlier (25)

Gamma globulin Human γ globulin (Fraction II) 12 per cent solution was purchased from AB Kabi Stockholm Sweden Rabbit γ globulin was prepared by ammonium sulphate precipitation of whole inactivated rabbit serum. The protein concentrations were determined by a modified Folin-Ciocalteau method (15) using the γ globulin from AB Kabi as standard

Denatured γ globulin preparations were obtained by heating one per cent solutions at 63° C for 10 minutes

Erythrocytes Human erythrocytes (O R_1R_1) were obtained from whole blood collected in sterile acid-citrate dextrose solution Sheep erythrocytes were obtained from whole blood collected in Alsever s solution Before use the erthrocytes were washed four times in 6-10 volumes of phosphate buffered saline pH 7.2 They were finally packed at 1 000 × g for 5 minutes

Formalinized sheep erythrocytes were prepared following the method described by *Daniel et al* (6) To one volume of packed erythrocytes were added eight volumes of a three per cent solution of formaldehyde in buffered saline The mixture was gently agitated for 24 hours at 4° C. Thereafter two volumes of 40 per cent formaldehyde were added and the mixture was again agitated in the cold for 24 hours The formalinized erythrocytes were then washed with buffered saline until no formaldehyde was detectable in the washing fluid (5)

Stromata of sheep erythrocytes were obtained following lysis in distilled water (25)

Tests for Anti γ Globulin Factors

Slide tests The slide test with sensitized formalinized sheep erythrocytes described by *Milgrom et al* (17) was slightly modified A two per cent suspension of formalinized erythrocytes was mixed with an equal volume of rabbit anti sheep erythrocyte serum at a dilution corresponding to eight agglutinating units (The antiserum had a titre of 4000 against two per cent suspension of erythrocytes. Accordingly this serum was used for sensitization at the dilution of 1 500) After incubation for 30 minutes at 37° C the sensitized erythrocytes were thoroughly washed They were stored at 4° C as a two per cent suspension. Merthiolate was added to make a final concentration of 1 10 000

The serum to be tested was diluted 1 16 in buffered saline Sensitized formalinized erythrocytes were vigorously shaken to obtain an even dispersion One drop each of the diluted serum and sensitized erythrocytes were mixed on a slide using a wooden stick. The slide was left undisturbed for 15 minutes at room temperature and the degree of macroscopically visible agglutination was read in indirect light against a dark background Identical results were obtained with active and heat inactivated sera (17)

The latex (RA) test was performed as recommended by the manufacturer with reagents purchased from the Hyland laboratories Los Angeles, Calif., USA

Tube tests The Waaler Rose test was carried out as described elsewhere (25) Sheep erythrocytes were sensitized by anti sheep erythrocyte serum at a dilution four times higher than the agglutination titre Twofold serial dilutions of serum to be tested were prepared in 0.2 ml volumes and 0.2 ml of a 0.5 per cent suspension of sensitized erythrocytes was added to each tube The agglutination was recorded after the tubes had been left overnight at 4° C.

The tests with anti D sensitized human erythrocytes were performed as previously (see 19) Erythrocytes were sensitized with an excess of incomplete anti D antibody They were tested with rabbit anti human globulin serum and reference anti Gm sera (19) Each serum was tested against erythrocytes sensitized by the polyspecific anti CD Ripley and by anti D specific for Gm(a) (and Cm(x Gm(b) and Cm(f) respectively For screening purpose the sera were tested in dilutions 1 4 1 16 and 1 64 otherwise serial twofold dilutions were prepared One drop each of serum dilution saline and one per cent suspension of sensitized erythrocytes were mixed and left at room temperature for one hour Before reading the tubes were centrifuged at 1 0000 × g for 30 seconds

The agglutination was recorded by gently agitating the tubes, the clumping was graded as +++, ++ or + at the moment when all erythrocytes were dislodged from the bottom of the tube

The titre of a serum was defined as the reciprocal of the highest serum dilution which gave at least + agglutination One agglutinating unit was defined as the minimum amount of serum which gave this agglutination

Latex fixation test in tubes (24) was performed with reagents purchased from Difco Laboratories, Detroit, Mich USA

Inhibition of agglutination The γ globulin preparations were properly absorbed by erythrocytes or by sensitized stromata (16) or by both in order to remove agglutinating activities against unsensitized or sensitized erythrocytes One volume of rheumatoid serum containing 16 agglutinating units was mixed with one volume of one per cent γ globulin solution After one hour of incubation at room temperature the sensitized erythrocytes were added Agglutination was recorded in the usual manner

RESULTS

Comparison of the Waaler-Rose Test Performed in Tubes and on Slides

Five hundred consecutive sera were examined by the Waaler-Rose tube test and by the slide modification All the 178 sera that gave strongly positive reactions in the slide test were positive in the tube test (Table 1) Of these, 156 gave strong reactions with titres of 128 or higher Of the 167 sera showing weakly positive reactions in the slide test, only 28 gave strong reactions in the tube test

TABLE 1

Comparison of the Waaler Rose Tube Test with the Slide Test

Results of the slide test	Titres in the Waaler Rose tube test				Total
	≦ 16	32/64	128/256	≧ 512	
—	148	5	2	0	155
± +	85	54	24	4	167
++, +++	0	22	81	75	178
Total	233	81	107	79	500

On the other hand, out of the 155 sera giving no reactions in the slide test seven gave positive reactions in the tube test, and two of these gave strong reactions Whether or not the lack of reaction with these sera in the slide test was due to inadequate incubation was investigated by placing the slides in wet chambers at room temperature However, there was still no reaction after two hours of incubation

The results obtained with these seven sera in other tests are shown in Table 2 The titres in the Waaler-Rose test varied considerably in repeat tests The agglutination scores were also low in relation to the titres The reactions were very dependent on the degree of sensitization, erythrocytes sensitized by half the amount of rabbit antiserum

used in standard test gave no reactions with any of the sera Accordingly these sera belonged to the group of rheumatoid sera exhibiting very "high slopes" (23, 25)

Two of the seven sera (3791 and 3821) gave strong reactions with anti CD Ripley sensitized erythrocytes (Table 2), but they did not react with erythrocytes sensitized by any of the other anti-D sera Four of the sera were negative both in the tube and the slide latex tests Two of these (3791 and 3923) were from patients who had suffered from definite rheumatoid arthritis for several years

Considering the various reactions recorded with the seven sera, we concluded that the agreement between the results obtained by the Waaler Rose test in tubes and on slides was satisfactory

TABLE 2

Reaction Patterns of the Seven Sera which were Negative in the Slide Test and Positive in the Waaler Rose Tube Test

Serum	Titres in Waaler Rose test	Reaction in Latex RA test	Titres in anti CD Ripley test
3271	256	+	<4
3791	64	—	512
3820	32	—	<4
3821	64	+	256
3923	256	—	<4
3945	64	+	<4
3962	64	—	4

Comparison of the Waaler-Rose Test with the Anti CD Ripley Test

The whole panel of sera, including the 500 sera presented above, was screened in both tests Serum dilutions 1 4, 1 16 and 1 64 were prepared in buffered saline using active sera Because prozones may occur with some sera, all three dilutions were tested with anti CD Ripley sensitized erythrocytes Only the dilution 1 16 was tested with sensitized formalinized erythrocytes The sera which gave positive reactions in this latter test were titrated in the Waaler-Rose tube test The sera were grouped according to the titres, those which gave no reactions in the slide test were considered < 16 The results are shown in Table 3

Out of the 626 sera which gave no reactions in the Waaler-Rose test, only five showed strong reactions in the anti CD Ripley test, and 50 gave weak reactions On the other hand, out of the 867 sera which gave no reactions in the anti CD Ripley test, as many as 92 gave strong reactions in the Waaler-Rose test and 204 gave weak reactions Accordingly the sera could be divided into four groups 1) sera negative in both tests 2) sera positive in both tests, 3) sera positive in the

of the reactions obtained in the Waaler-Rose tube test with these sera is therefore doubtful Accordingly the slide modification is well applicable for the routine serological work, saving a lot of time.

As suggested earlier (17), parallel performance of this test and the latex test would appear advisable since the latter test is more sensitive Although the specificity of the latex test is reduced, for instance sera from elderly healthy persons give positive reactions (10, 13, 26), the latex test will detect most of the rheumatoid factors

The test with polyspecific incomplete anti-Rh (diagnostic) as performed in the present work, is also simple and saves precious reagents By this procedure it is possible to search for various isospecific anti-γ-globulin factors

Using these screening methods, three different types of reactions were distinguished in the 1200 sera tested Some sera reacted only with human γ-globulin, some reacted only with rabbit γ-globulin and other reacted with γ globulin of both species It should also be noted that some sera which reacted with anti CD Ripley sensitized erythrocytes did not react with human γ-globulin in the latex test The reaction was inhibited by pooled human γ-globulin Hence the factor responsible for the reaction was considered to be a rheumatoid factor Further investigations of this factor are currently in progress

In recent years controversial reports have appeared concerning the molecular basis for the specificity of the rheumatoid factor reacting with rabbit γ-globulin (see 12) Some authors (2, 3, 4) claim that this reaction is caused only by cross-reacting molecules, while other authors (16, 18, 27) recognize separate molecules with primary specificity for rabbit γ-globulin The results of this work strongly support the latter view Agglutination obtained with sera which showed strong reaction in only one of the two tests, i e reaction with either human or rabbit γ globulin was inhibited by homologous γ globulin only These sera can therefore be considered as monospecific In addition some of the sera which reacted strongly in both tests also showed mainly species restricted inhibition With other sera the cross reaction was predominant Accordingly the proportions of rheumatoid factor molecules of any specificity vary from individual to individual In another study it was recently shown that other anti-γ-globulin factors also were heterogeneous and showed similar individual variations (20, 21)

A further question of interest is the relation of the various factors to the clinical development of the disease A factor directed against rabbit γ-globulin without evidence of reactivity toward human γ-globulin has been found in a human serum following vaccinations (1) If rheumatoid factor is considered an antibody to autologous γ-globulin, the question how the specificities arise is intriguing The authors believe that rheumatoid sera exhibiting monospecific reactions will be best suited for future investigations concerning the biological meaning of the specificities of various anti-γ-globulin factors

SUMMARY

The slide modification of the Waaler Rose test using formalinized sheep erythrocytes was compared with the standard tube test Good agreement of results was observed in testing 500 sera of patients sus pected of suffering from rheumatoid arthritis Only seven sera showed discrepancies giving no reaction in the slide test and weak reaction in the tube test In the routine serological work it is therefore sufficient to titrate in the standard tube test only those sera which are positive in the slide test

Twelve hundred sera were screened for anti γ globulin factors using the Waaler Rose slide test and a tube test with human erythrocytes sensitized by incomplete anti CD (Ripley) Three different types of re- actions were distinguished Some sera reacted only with human γ glo- bulin some reacted only with rabbit γ globulin and other reacted with γ globulin of both species Inhibition experiments with human and rabbit γ globulin indicated that the monospecific sera contained rheum- atoid factor molecules of one specificity only, directed either against human or against rabbit γ globulin

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DISTRIBUTION OF THE ABO, MN, Rh, DUFFY AND KELL BLOOD GROUPS IN A RANDOM SAMPLE OF SWEDISH MEN AGED FIFTY

By

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The variation of blood groups and gene frequencies in Sweden have been studied mainly in blood donors, conscripts, and in paternity series Blood donors are often selected and cannot *a priori* be considered as representative of the total population In groups of conscripts misclassifications have been a problem In regard to paternity series it has been questioned whether selective mechanisms might be active in the mothers, at least within certain blood group systems (11) In Sweden regional variations of blood group frequencies within different systems have been demonstrated, *e g* by *Beckman* (1) and *Heiken* (3 6)

In the present study, a random sample of men born in 1913 and living in Gothenburg has been thoroughly examined It has been possible to analyse five blood group systems in the same material The findings have been compared with those obtained in some recent Swedish investigations

MATERIAL

During 1963 a population study of men born in 1913 on days of the month divisible by 3 was performed at the Sahlgren Hospital in Gothenburg In order to eliminate the age factor men of the same age were examined It was a 4 hour investigation of the individual s state of health

The study is described in detail elsewhere (14) 855 participants (88 per cent) were examined in the hospital Of the participants 624 (73 per cent) were born in Gothenburg or nearby 187 (22 per cent) in other parts of Sweden and finally 44 (5 per cent) in foreign countries

The non participation group was analysed separately (15)

Serological Techniques

ABO The red cells of the subjects were tested with anti A and anti B and a check of iso agglutinins was performed with A_1 A_2 and B test cells

MN Commercially available anti M and anti N sera (Dade reagents Miami Fla and Hyland Lab Calif., USA) were employed The slide methods were used according to the manufacturers instructions Checks of the MN groups by means of absorption procedures were not performed MN determination was carried out on 843 of the samples

Rh Determinations were performed with five antisera (anti C+Cw anti Cw anti D anti E anti-c and anti e) by the papain method of *Löw* (8) All D negative samples were tested for the possible presence of the Du antigen by means of potent incomplete anti D using the indirect anti globulin technique

Kell and Duffy Incomplete anti k and anti Fya sera were used employing the indirect anti globulin technique

Statistical Methods

ABO The gene frequencies p q and r were calculated by means of the corrected formulae given by *Bernstein* (2) To test the goodness of fit between the observed and expected phenotype frequencies the formula proposed by *Stevens* (13) was used

MN The M and N gene frequencies were calculated by means of gene counting (10 12) The agreement between expected and observed frequencies was tested with Fisher s χ^2 test for MN results (cited by *Race & Sanger* 12)

Rh Calculations have been performed according to the formulae given by Mourant (10) for surveys with five antisera Indications that the gene complexes *CdE CwdE Cwde* or *CwDE* occur in the material were not found

Kell The frequency of the genes was calculated as

$$k = \sqrt{kk} \text{ and } K = 1-\sqrt{kk}$$

A statistical check of the genetical reliability is not possible

Duffy Since the samples were tested with anti Fya but not with anti Fyb possible instances of the phenotype Fy(a b) could not be recognized hence the gene Fy responsible for this phenotype was not taken into account in the calculations. The gene frequencies were estimated as for k and K

Comparison of the materials has been performed by calculation of heterogeneity χ^2 according to the formula proposed by Fisher (cited by *Beckman* 1)

$$\chi^2 = \frac{1}{p\ q}\ (S(ap)\ n\ \bar{p}) \quad \text{where } q = 1\ p$$

RESULTS AND CONCLUSIONS

The blood group and gene frequencies obtained for the different systems are summarized in tables 1 6 For purposes of comparison we have also included in the tables the results of some recent Swedish studies on the different blood group systems performed by *Beckman* (1) *Heiken* (3 4 5 6) and *Low* (9)

The figures obtained for the ABO system indicate that the genetical reliability is good ($\chi^2 = 0\ 6886$ 1 d f $0\ 5 > p > 0\ 3$) (cf Table 1)

TABLE 1

Blood Group and Gene Frequencies within the ABO System

	A	B	0	AB	Total	χ^2	p	q	r	p+q+r
Present material number	396	86	341	32	855	0 6886	0,2938	0 0717	0 6344	0 9999
%	46 32	10 06	39 88	3 74						
Beckman (1)* number	4939	1040	3974	504	10457	0 277	0 3073	0 0768	0 6159	1 0007
%	47.23	9 95	38 00	4 82						

* Paternity material (Table 105)

TABLE 2

Blood Group and Gene Frequencies within the MN System

	MM No	MM %	MN No	MN %	NN No	NN %	Total	χ^2	Genes % M	Genes % N
Present material	274	32.50	416	49.35	153	18.15	843	0.05	57.18	42.83
Heiken (5)	198	31.18	302	47.56	135	21.26	635	0.9839*	54.96	45.04
Beckman (1)**	3370	32.23	5178	49.52	1909	18.26	10457	0.002	56.99	43.01

* Calculation based on the figures given by Heiken (5)
** Table 112

TABLE 3

The Frenquency of Genotypes within the Rh System

Genotype according to *Race & Sanger* (12)	Genotype according to *Wiener & Wexler* (16)	Present investigation: Observed blood groups No	Present investigation: Observed blood groups %	Present investigation: Calculated genotype frequency %	*Heiken* (6): Observed blood groups %	*Heiken* (6): Calculated genotype frequency %
cde/cde	rr	140	16.37	17.15	15.65	15.30
cDe/cde	R^0r	13	1.52	1.56	1.65	1.57
cDe/cDe	R^0R^0			0.04		0.04
cdE/cde	$r''r$	1	0.12	0.12	0.28	0.27
cdE/cdE	$r''r''$	0	0.00	0.00	0.00	0.00
cDE/cDE	R^2R^2	25	2.92	2.93	2.83	2.60
cDE/cdE	R^2r''			0.05		0.11
cDE/cde	R^2r	141	16.49	14.19	12.71	12.62
cDE/cDe	R^2R^0			0.64		0.65
cDe/cdE	R^0r''	--		0.01	--	0.01
Cde/cde	$r'r$	4	0.47	0.47	0.40	0.40
CDe/cde	R^1r	279	32.63	30.82	32.59	31.10
CDe/cDe	R^1R^0			1.40		1.60
cDe/Cde	R^0r'			0.02		0.02
C^wDe/cde	$R^{1w}r$	8	0.94	1.19	1.58	1.62
C^wDe/cDe	$R^{1w}R^0$			0.05		0.08
cdE/Cde	$r''r'$	0	0.00	0.00	0.00	0.00
CDe/cDE	R^1R^2	96	11.23	12.75	13.66	12.83
cDe/CDE	R^0R^z			0.01		0.00
CDe/cdE	R^1r''			0.11		0.27
cDE/Cde	R^2r'			0.20	--	0.16
CDE/cde	R^zr			0.17		0.05
cDE/CDE	R^2R^z	1	0.12	0.07	0.00	0.02
cdF/CDE	$r''R^z$			0.00		0.00
C^wDe/cDE	$R^{1w}R^2$	8	0.94	0.49	0.70	0.67
C^wDe/cdE	$R^{1w}r''$			0.00		0.01
Cde/Cde	$r'r'$	0	0.00	0.00	0.00	0.00
CDe/CDe	R^1R^1	129	15.09	13.85	16.07	15.81
CDe/Cde	R^1r'			0.42		0.41
C^wDe/CDe	$R^{1w}R^1$	9	1.05	1.07	1.82	1.65
C^wDe/Cde	$R^{1w}r'$			0.02		0.02
C^wDe/C^wDe	$R^{1w}R^{1w}$	--	--	0.02	--	0.04
CDe/CDE	R^1R^z	1	0.12	0.16	0.06	0.06
Cde/CDE	$r'R^z$			0.00		0.00
CDF/CDE	R^zR^z	0	0.00	0.00	0.00	0.00
C^wDe/CDE	$R^{1w}R^z$	0	0.00	0.01	0.00	0.00
		855	100.01	99.99	100.00	99.99

TABLE 4

The Frequency of Rh Chromosomes

Chromosome	Present investigation	Heiken (6)
CDe	0.3721	0.3976
C^wDe	0.0144	0.0207
cde	0.4141	0.3911
cDE	0.1713	0.1613
cDe	0.0188	0.0201
Cde	0.0057	0.0051
cdE	0.0015	0.0034
CDE	0.0021	0.0007

TABLE 5

Blood Group and Gene Frequencies within the Kell System

	Phenotypes				Total	Genes %		Genotypes %		
	K+		K−							
	No	%	No	%		K	k	KK	Kk	kk
Present material	78	9.12	777	90.88	855	4.69	95.31	0.22	8.95	90.83
Heiken (3)	322	7.11	4205	92.89	4527	3.62	96.38	0.13	6.98	92.89
Löw (9)	50	7.1	650	92.9	700	3.6	96.4	0.13	6.94	92.93

Comparison of our results with those obtained in Beckman's total paternity material indicates no significant difference in blood group frequency ($\chi^2 = 4.5$, 3 d.f., $0.3 > p > 0.2$). Neither does comparison with Beckman's results for regions VIII and/or X, those which most closely compare with the Gothenburg area, reveal any statistically significant differences. The low frequency of group AB is in accordance with the statement by *Mourant* (10) who declared that "there will always tend to be an apparent deficiency of this group".

The frequencies found within the MN system (Table 2) are in accord with those reported by *Beckman* (1). They are also in good agreement with those given by *Heiken* (5). The χ^2-value 0.05 (Table 2), calculated from our figures, indicates a good consistency of the material ($0.90 > p > 0.80$, 1 d.f.).

A calculation of the expected Rh phenotypes revealed a pronounced excess of ccDEe individuals, in the total material, however, $0.2 > p > 0.1$. It is probable that this fact may be responsible for the difference between *Heiken's* (6) and our series. This difference, however, is not statistically significant ($\chi^2 = 13.9$, 7 d.f., $0.10 > p > 0.05$). The frequency of Rh chromosomes in *Heiken's* (6) and the present material are recorded in Table 4.

The Kell blood group frequencies reported by *Heiken* (3) and *Löw* (9) are identical (Table 5). The frequency of K+ individuals in the present study is somewhat higher and differs significantly from that

TABLE 6

Blood Group and Gene Frequencies within the Duffy System

	Phenotypes					Genes		Genotypes		
	Fy(a+)		Fy(a—)			%		%		
	No	%	No	%	Total	Fy^a	Fy^b	Fy^aFy^a	Fy^aFy^b	Fy^bFy^b
Present material	584	68 30	271	31 70	855	43 70	56.30	19 10	49 20	31 70
Heiken (4)	229	66 57	115	33 43	344	42 18	57 82	17 79	48 78	33 43

reported by *Heiken* (3) ($\chi^2 = 4\ 18$ 1 d f, $0\ 050 > p > 0\ 025$) Our figures are in better agreement with those reported by *Heistö* (7) for 1000 citizens of Oslo, and by *Race & Sanger* (12) for an English population

The figures obtained for the Duffy system are in accord with those reported by *Heiken* (4) ($\chi^2 = 0\ 10$, 1 d f, $0\ 80 > p > 0\ 70$)

As already described, one fourth of the participants were born outside the Gothenburg region However, no significant differences within any of the systems tested could be demonstrated whether the groups were compared with each other or were compared with the total material The inclusion of the groups of men born outside the Gothenburg region has probably not influenced the results obtained for the total material

SUMMARY

The blood group and gene frequencies within the ABO, MN, Rh, Kell and Duffy systems have been determined in a random sample of 855 middle-aged Swedish men living in Gothenburg When the results obtained were compared with those reported earlier for Swedish populations only the Kell system was found to differ significantly

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INVESTIGATIONS ON THE ENZYMES AND TOXINS OF STAPHYLOCOCCI

Study of the "Egg Yolk Reaction" Using an Agar Plate Assay Method

By

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Received 14 iii 66

The closely associated occurrence in staphylococci of coagulase and an enzyme causing opacity in egg yolk was first described by *Gillespie & Alder* (4) It was shown that this reaction on egg yolk was due to a lipase which was active against lard but not against tributyrin if used as the substrate On the other hand, *Drummond & Tager* (1) showed that purified staphylocoagulase preparations contained an esterase which liberated acid from both egg yolk and tributyrin and these results seemed at first to suggest that coagulase and the esterase could be identical *Drummond & Tager* (2) further showed that these two activities were separable entities and distinct from yet another hydrolytic enzyme, acid phosphatase (3) However, the nature of the reaction if egg yolk is used as the substrate is not fully understood as yet

The association of coagulase and the egg yolk enzyme was confirmed by *Lundbeck & Ask* (5, 6) in a study on strains isolated from food Because the egg yolk enzyme or the products of its activity may be connected with the occurrence of enterotoxin in food stuffs, by analogy with the Clostridium perfringens enzyme which liberates phosphorycholine (7), the study of the egg yolk reaction itself is of great interest and importance

The methods hitherto used for the determination of the enzymes attacking egg yolk are both cumbersome and inaccurate A new simplified and more accurate plate method, especially convenient for the quantitative assay of the egg yolk enzyme, was presented by the authors in a preliminary communication (9) This method has been found convenient for routine diagnostic purposes as well as for studies on the nature of the egg yolk reaction It can be used also for the demonstration and quantitative assay of antilipase antibodies The present communication describes studies on the egg yolk enzymes found in colonies growing on agar surface as well as in liquid cultures



Fig 1
Reacti n given bv staphylccocci on egg yolk agar after 24 hours growth

MATERIAL AND METHODS

Strains A total of 395 phage typed clinical isolates of *Staphylococcus aureus* obtained from the Bacteri logical Department of the National Bacteriological Labora tory and 8 lab rators strains were studied The typing scheme empl yed was as follows

Gr up I	29 52 52A 79 80 81 KS6
Group II	3A 3B 3C 55 71
Group III	6 7 42E 47 53 54 75 77 83 83A
Group IV	42D
	187
NT	non typable strains

The cultures were store l on *nutrient* agar stabs at 4° C and were streaked out on nutrient agar plates when neede l

Egg yolk reaction of growing cultures For the direct demonstration of the reaction of growing cultures the organisms were plated out on egg yolk agar plates of the

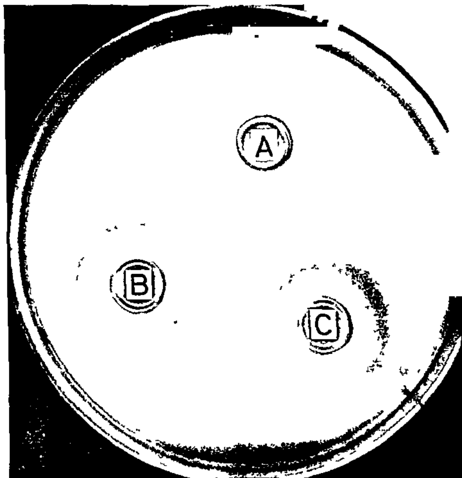


Fig 2

Reaction obtained with cell free culture supernatant on egg yolk agar plate Well A contains saline and gives no reaction well B and C contain supernatant filled once and twice respectively A region of clearance and precipitation can be noticed

following composition (5 6) Beef extract 5 5 g peptone 10 g NaCl 3 g Na_2HPO_4 $12H_2O$ 2 g *aqua dest 1000 ml Difco agar 15 g pH adjusted to 7 6* The mixture was steamed to dissolve the agar filtered through gauze and sterilized at 120° C for 30 minutes After cooling to 50° C sterile egg yolk was added to a final concentration of 1 25 per cent by volume unless otherwise stated the sodium azide was added to a concentration of 0 015 per cent The pH was checked before pouring into Petri plates and care was taken to assure an even distribution of egg yolk solids The organisms were streaked on the egg yolk agar plates and incubated for three days at 37° C The cultures were examined for egg yolk reaction, morphology of the colonies and for pigmentation (Fig 1)

Egg yolk reaction of cell free culture supernatants The staphylococci were grown for five days in Difco brain heart infusion broth at 37° C then centrifuged at 30 000 g for 20 minutes at 4 C Sodium merthiolate was added as a preservative to the supernatant to a final concentration of 0 01 per cent In order to remove remaining cells the supernatants were Seitz filtered Egg yolk agar plates containing 0 01 per

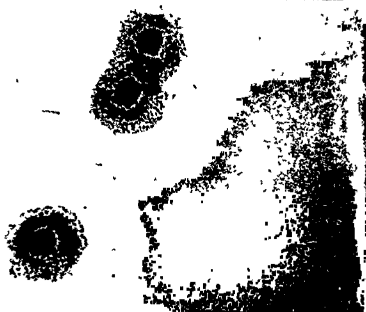


Fig 5

Liberation of fatty material on the agar surface by cultures growing on egg yolk agar

seen in growing cultures was then investigated and the experimental conditions yielding the best results were determined

The reaction obtained when staphylococci were grown on egg yolk agar plates is represented in Fig 1 A gross examination showed a zone

of clearance around the colonies A closer examination of the colonies (Figs 3 and 4) revealed that the clinical isolates presented a wide variety of morphologic characteristics from translucent to opaque colonies and from rounded to hexagonal heptagonal or octagonal forms The formation of a spreading translucent or whitish material around the colonies of some strains was also observed (Fig 4 top left and bottom) When the centre of such colonies was pigmented deeply yellow they assumed the appearance of *fried eggs*

The pigmentation of the colonies varied from colourless or whitish to lemon yellow and yellow orange colour Against the white background of egg yolk agar the pigmentation could be readily assessed

The egg yolk reaction given by the clinical isolates was further characterized by a clear zone formed around the colonies Within the clear region and close to the colonies a zone of precipitation was observed The extent of such precipitation and the size of the clear zone were not always proportional with different strains and thus they appeared to be independent yet closely associated phenomena On the surface above the region of precipitation a layer of glistening fatty material appeared which could be observed if the plates were viewed from an angle (Fig 5) No specific correlation between colony morphology and egg yolk reaction was demonstrable

Egg Yolk Reaction of Culture Supernatants

When holes cut in the egg yolk agar medium were filled with the cell free culture supernatants and incubated for 24 hours a clear zone and a region of fine precipitation similar to that given by growing cultures were obtained (Fig 2) Refilling and further incubation for another 24 hours increased the zone diameter and concentric rings of lysis and precipitation were formed The experiments performed up to five days yielded several concentric rings depending apparently upon the number of refillings A layer of fatty material similar to that given by cultures growing on egg yolk agar plates also appeared over the precipitation region

The Rate of Reaction and the Effect of Different Amounts of Enzyme and Substrate

The rate of the egg yolk reaction with repeated daily refillings was studied by plotting the diameter of the zone of reaction obtained on different days against the time of incubation in days As indicated in Fig 6 a linear relationship was established

The relationship between the rate of reaction and the concentration of the egg yolk enzyme was standardized by converting the enzyme concentration to "units of activity Using different concentrations of a highly active supernatant a linear relationship was obtained when the

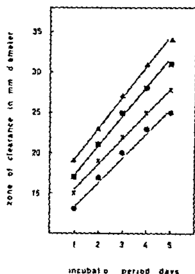


Fig 6
Rate of egg yolk reaction by culture supernatants

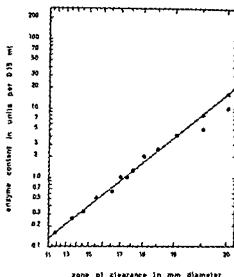


Fig 7
Standard curve for the egg yolk reaction

logarithmic concentration of the egg yolk enzyme was plotted against the logarithmic increase in the diameter of reaction (Fig 7) One unit of the egg yolk enzyme is defined as the amount enzyme contained in 0 15 ml giving a 17 mm diameter zone of clearance after 24 hours incubation at 37° C under the standard conditions described under the head of material and methods

The reaction obtained with culture supernatants in the presence of different concentrations of egg yolk (0 125, 0 25, 0 50, 0 75, 1 00, 1 25, 2 50 and 5 00 per cent) indicated that the optimum concentration ranged between 0 75 and 1 25 per cent Above 1 25 per cent the zone of reaction tended to diminish and below 0 75 per cent it was diffuse and ill defined 1 25 per cent egg yolk was the highest concentration which gave optimal results, especially when the reaction was studied after several refillings

Effect of pH on the Egg Yolk Reaction

The effect of pH on the egg yolk reaction was studied by varying the initial pH of the egg yolk agar before it was poured into plates, and adjusting the pH of the culture supernatants to coincide with that of the agar on which they were tested The pH-activity relationship, shown in Fig 8, indicated that the reaction was maximal at pH 7 6 Above this pH the reaction was diffuse because the egg yolk solids tended to dissolve Between pH 5 0 and 6 5 a precipitation reaction was obtained

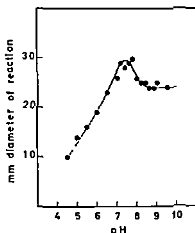


Fig 8

Egg yolk reaction pH – activity relationship
--- --- = precipitation only ⇐ diffuse reaction

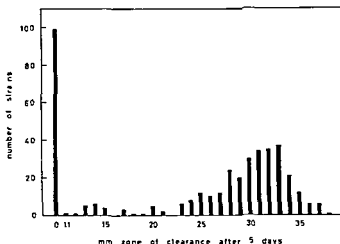


Fig 9

Occurrence of egg yolk enzyme among 402 staphylococcal isolates

Egg Yolk Reaction of the Supernatants of Clinical Isolates

The activity given by supernatants from clinical isolates and cultures of laboratory strains on egg yolk agar plates is shown in Fig 9 A large number of strains (99 in number representing 25 per cent of the total) did not liberate any detectable amount of the enzyme in the supernatant While about 30 cultures gave rather low activity (below 22 mm diameter), the supernatants of most of the remaining strains gave high activity (23 to 38 mm diameter)

Relation of phage type to occurrence of egg yolk enzyme No specific

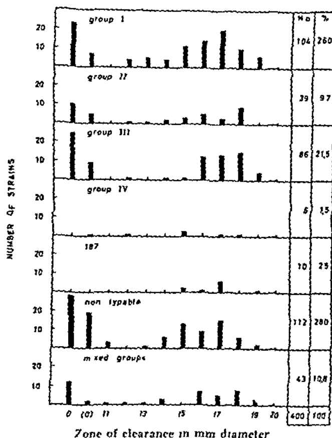


Fig 10
Occurrence of egg yolk enzyme among staphylococci of different phage groups

correlation between the occurrence of egg yolk enzyme among the clinical isolates and the distribution of phage types was found (Fig 10) Cultures with various grades of activity were found to occur in all phage and non-typable groups

DISCUSSION

The egg yolk reaction as described by Gillespie & Alder (4) was characterized by the formation of opacity in egg yolk on incubation with culture supernatants These workers used egg yolk diluted in saline and the reaction mixture of the tests was clear They did not detect the *initial clearing reaction which was observed when the tests were done* by the present plate method using egg yolk solids precipitated in the *form of a fine suspension* The egg yolk reaction observed, either by a *study on growing staphylococci on egg yolk agar plates or by tests of culture supernatants, was found to consist of three phenomena a primary* lytic reaction, a precipitation reaction and the liberation of fatty material These three phenomena may be caused by the action of more

than one enzyme in the supernatants on a variety of substrates in egg yolk These complex materials need to be fractionated further before the nature of the substrates and the enzymes involved can be established

In the present study the extent of precipitation and clearing was found to vary in different strains This observation suggests that the two phenomena may be independent However, the liberation of fatty material can be due to the action of a lipase or an esterase, which would be in agreement with the results obtained by *Gillespie & Alder* (4) as well as with those obtained by *Drummond & Tager* (1) Studies by *Shah & Wilson* (8) also indicated that the substrate in egg yolk was lipovitellenine and that the opacity reaction was due to a lipase

With the egg yolk agar plate method a pH optimum of 7 6 was obtained, and the formation of the precipitate alone was noticed below pH 6 5 *Gillespie & Alder* found a pH optimum of 5 5 for the opacity reaction while *Shah & Wilson* found also an optimum at pH 5 5 and another at pH 8 0 The latter workers suggested that the optimum noticed at pH 5 5 could be due to the lability of lipovitellenine at this pH It was also possible that the use of glucose together with egg yolk saline in the cultures might result in production of acid which in turn could precipitate the protein

Besides indicating the clearing and precipitation reactions in a quantitative manner, the use of egg yolk agar plates containing sodium azide, also permits the selective cultivation of staphylococci from heavily contaminated material

SUMMARY

The egg yolk reaction was studied using an egg yolk agar plate assay method The reactions obtained by growing staphylococci on such plates were also given by their cell free culture supernatants and optimal results were obtained when the egg yolk concentration was between 0 75 and 1 25 per cent The egg yolk reaction was composed of three phenomena—a clearing reaction, formation of a precipitate and the liberation of fatty material A pH optimum of 7 6 was obtained for the reaction The reaction was measured as a change in the diameter of the zone of clearance which increased linearly with time and enzyme concentration

Interesting colony morphologic characteristics were noted when 403 isolates were cultivated on egg yolk agar plates No specific correlation between colony morphology, phage group and occurrence of egg yolk enzyme activity was obtained

The plate method was a convenient means for the qualitative and quantitative estimation of the egg yolk reaction and permitted the selective cultivation of staphylococci from heavily contaminated material

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INVESTIGATIONS ON THE ENZYMES AND TOXINS OF STAPHYLOCOCCI

Proteolytic Enzymes and Their Purification

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The proteolytic enzymes of the staphylococci have not been studied to the same extent as other bacterial or mold proteases (8) Most of the studies on these staphylococcal enzymes (6, 7, 12, 13, 18, 19, 21) correlated the property of growing cultures or culture supernatants to liquefy gelatin or digest other protein substrates to the occurrence of other products such as coagulase or α haemolysin

Abe (1, 2) investigated the pH activity relationship of crude enzyme preparations using proteins and peptides as substrates *Robinson et al* (20) discovered the closely associated occurrence of proteolytic activity with α-haemolysin which they purified by column chromatography and suggested that these two were identical

The staphylococci also produce a kinase, like streptokinase, which activates plasminogen The production of this and other proteolytic enzymes in staphylococci may greatly aid in their multiplication in tissues and other organs, but the rôle these enzymes play in infection is not established Their importance to the pathogenicity of the bacteria the occurrence of antibodies to these enzymes and their protective effect against infection are still to be investigated In order to study these aspects it is essential to have a purified enzyme preparation which is free from the two factors generally regarded pathogenic, coagulase and α-haemolysin The present investigation describes the purification and some properties of the proteolytic enzymes

MATERIAL AND METHODS

Strain and cultivation Staphylococcus aureus strain Walker phage type 42D was stored in the lyophilized state The organism was grown for three days at 37° C on a casein hydrolysate medium with the continual passage of 25 per cent CO_2 and 75 per cent air as described earlier for α haemolysin production (22) The culture was centrifuged at 30 000 g and the supernatant was Seitz filtered

Determination of Proteolytic Activity on Different Substrates

Gelatin The rate of hydrolysis of gelatin was measured viscosimetrically as described by *Hultin* (9, 10) and further applied by *Hultin & Lundblad* (11 16, 17)

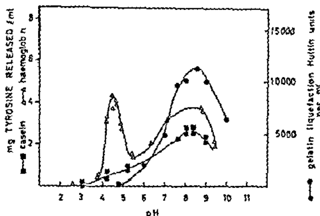


Fig 1

pH activity relationship for the hydrolysis of gelatin haemoglobin and casein by staphylococcal protease

One ml of the test sample was mixed with 3 ml of a 4 per cent gelatin (U SP granular Fisher) solution in 0 1 M of different buffers containing 0 01 per cent merthiolate as a preservative The flow time of the mixture was measured at 35 5° C in an Ostwald viscosimeter and the gelatinase activity calculated according to Hultin's formula

Casein Four grams of casein nach Hammarsten (E Merck Darmstadt) 4 ml of 0 1 M NaOH and 1 ml of 1 per cent merthiolate (Schmitt-Jourdan France) was made up to 100 ml with distilled water The proteolytic activity in the test sample was determined according to the method of *Kunit* (14) The incubation mixture contained 2 ml of 0 4 M buffer 1 ml of 4 per cent casein and 1 ml of the enzyme solution The mixture was incubated at 35 5° C for 4 hours in a shake incubator The reaction was stopped by adding 5 ml of 0 3 M trichloracetic acid after 30 min at 35 5° C the mixture was filtered and the liberated tyrosine in the filtrate was estimated at 280 mμ Controls without enzyme were run in parallel and the enzyme was added after the incubation time

Haemoglobin Urea denatured bovine haemoglobin (Enzyme Substrate Powder Armour England) was used The proteolytic assay was done according to the method of *Anson* (3) The incubation mixture consisted of 2 0 ml of 0 4 M buffer 1 0 ml of 4 per cent haemoglobin and 1 0 ml of enzyme solution The mixture was kept at 35 5° C for 4 hours in a shake incubator and then treated as described for casein Blanks were run in parallel

EXPERIMENTAL

Optimal conditions such as pH for the determination of proteolytic activity with the different substrates and the stability of the enzyme at various temperatures were determined Using these substrates the location of the enzyme in the various fractions during purification was studied

pH-Activity Relationship

The activity of the crude enzyme at various pH values on the different substrates is shown in Fig 1 With gelatin as the substrate high activity was noted between pH 7 5 and 9 0 with a maximum at 8 6 A similar pH activity relationship was found to occur with casein as the substrate, but the pH optimum was at 9 5 With haemoglobin as the

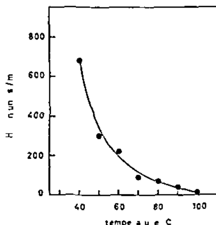


Fig 2
Effect of exposure to various temperatures on the stability of staphylococcal protease for gelatin hydrolysis

substrate two pH optima were demonstrable one at pH 4 5 and the other between 8 0 and 9 0 The amount of tyrosine released from haemo globin at alkaline pH was almost twice that liberated from casein The occurrence of two pH optima for the hydrolysis of haemoglobin sug gested the possibility that two enzymes might be involved

Stability of Enzyme after Exposure to Various Temperatures

Aliquots of an ultrafiltered concentrate of the culture supernatant were exposed to various temperatures for 30 minutes in a water bath then cooled and stored at 4° C before being tested for activity the next day Proteolytic activity in these samples was studied using gelatin as the substrate (Fig 2) It was found that the enzyme activity decreased gradually with increasing temperature Ten per cent of the activity was found after exposure to 70° C and little activity remained after boiling

Purification of the Enzyme by Gel Filtration on Sephadex G 100

It was suggested by *Robinson et al* (20) that the α-haemolysin and proteolytic enzyme were identical During purification of the former *the proteolytic activity of the different fractions was investigated* Chromatography on a column of Sephadex G 100 (Pharmacia Uppsala) was done as described earlier using 0 05 M TRIS-HCl pH 7 6 (22) Extinction measurements at 280 and 260 mμ showed the occurrence of five peaks (Fig 3 bottom) The proteolytic activity in the different fractions was studied using the three substrates As shown in Fig 3 (bottom) maximal activity with gelatin as the substrate occurred in tube number 48 and the entire activity appeared between tubes 40 and 60 The shape of the descending curve after tube 53 seemed to suggest either that two activities were occurring close together or that the effect

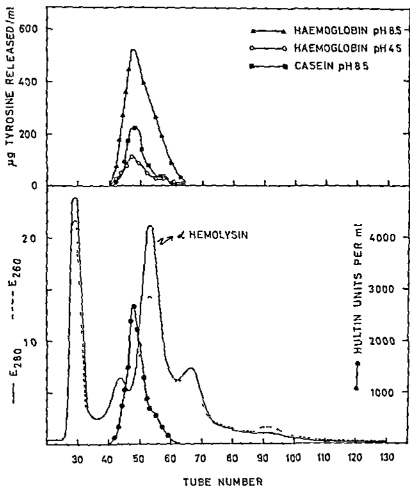


Fig 3

Fractionation of staphylococcal protease on Sephadex G-100

was due to tailing which is common in chromatographic experiments Tests with haemoglobin at pH 4 5 and 8 5 and with casein at pH 8 5 showed that all the proteolytic activity was concentrated within the fractions between tubes 40 and 60 with the peak in tube 48 (Fig 3, top)

Purification of Enzyme by Electrophoresis

The contents of tubes between 40 and 60 from the Sephadex G-100 chromatography were pooled, concentrated by ultrafiltration using collodion filters to a minimal volume and subjected to electrophoresis under conditions described earlier (22) using Sephadex G-25 as the supporting material and employing 0 01 µ Tris-HCl, pH 9 1 at 4° C Five components could be separated three of which had higher 260 than 280 mµ absorption while the other two showed the opposite relation

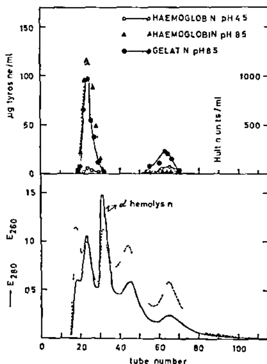


Fig 4

Separation of proteolytic enzymes and α haemolysin by electrophoresis

(Fig 4, bottom) Proteolytic activity with gelatin and haemoglobin as the substrates occurred in two peaks as shown in the upper figure Activity was noted in tubes between 20 and 30 with a maximum at 23 corresponding to the first peak giving higher 280 than 260 mμ absorption The fraction between tubes 55 and 71 contained lower proteolytic activity than the other The first fraction had a higher activity with haemoglobin as substrate at pH 8 5 than at 4 5 whereas the second had the opposite relation These results support the previous suggestion that the two pH optima noted with haemoglobin as the substrate are due to two different enzymes As noted earlier (22) α-hamolysin was found to occur in the second protein peak with high 280 mμ absorption between tubes 30 and 40 with the maximal activity in tube 32 which corresponds to the tube having the highest absorption

Thus the two proteolytic enzymes were separable from one another as well as from α haemolysin by electrophoresis

DISCUSSION

In the present study the proteolytic enzymes of a strain of Staph aureus had been investigated A pH optimum was found in the alkaline region

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From the Institute of Medical Microbiology, Department of Bacteriology, University of Uppsala, Sweden

STIMULATION OF STAPHYLOCOCCAL HAEMOLYSIN PRODUCTION BY LOW CONCENTRATIONS OF PENICILLIN

By

H O HALLANDER, G LAURELL and G LOFSTROM

Received 15 ii 66

It is known that penicillin acts on bacteria by interaction in the cellwall synthesis (7, 9) Less information is available, however, as to the direct influence on the metabolism of the bacteria There is some evidence that penicillin in low concentration might stimulate the growth of bacteria (8) *Elek* (2) also mentions that a zone of increased growth can often be observed beyond the edge of inhibition in plate sensitivity tests In our laboratory it has been observed on several occasions in performing the antibiotic sensitivity test on sheep blood agar plates that the penicillin discs were surrounded by a fairly narrow haemolytic zone at the border of the growth also in strains which were not haemolytic when growing freely on the medium This phenomenon has been studied in some detail in the present paper

MATERIAL AND METHODS

Strains of *Staphylococcus aureus* were selected from the daily routine examinations Twenty strains showing the typical haemolytic zone were chosen of which ten were resistant to benzylpenicillin and ten sensitive For each of these one strain with the corresponding sensitivity but without a haemolytic zone was isolated For the gel diffusion test a strain (13671) was used which was sensitive to penicillin but which showed no haemolytic zone

Antibiotics

[illegible] Astra Sweden lyophilized in lots of [illegible] penicillin sodium [illegible] d in the main ex [illegible] other antibiotics [illegible] −20° C diluted in [illegible] d in the dilution tests only once

Antibiotic Sensitivity Test

The disc diffusion method according to *Ericsson et al* (3) was applied for antibiotic sensitivity tests Ordinary sheep blood agar medium was used

Performance of the Tube Dilution Test

The medium used for the cultivation of bacteria for the toxin assays contained 0 3 per cent beef extract (Difco), 1 per cent peptone (Difco) and 0 3 per cent sodium chloride

The penicillin resistant staphylococci were cultured in a series of twofold serial dilutions of methicillin—from 10–0 009 μg/ml in this medium including a control culture without addition of the antibiotic The sensitive strains were cultivated in a corresponding dilution series of benzylpenicillin (0 25–0 015 IU)

For inoculation bacteria from a 48 hour blood agar plate were suspended in saline The density of the bacteria was corrected to an extinction of 0 7 + 0 05 in a Beckman C Colorimeter (green filter) 0 5 ml of bacterial suspension was inoculated into 10 ml portions of broth containing different amounts of penicillin 20 ml glass flasks with cottonplugs were used After 18 hours at 37° C on a rotary shaker the density of the cultures were measured in the colorimeter as above The suspensions were centrifuged for 15 min at 4500 r p m and the supernatants titrated for toxin activity

Toxin Assay

1) The alpha haemolysin titre was measured by incubating 0 5 ml of a 1 per cent suspension of washed rabbit erythrocytes with 0 5 ml of serial dilutions of the sample to be investigated The titrations were read after 1 hour at 37° C and 1 hour at room temperature The highest toxin dilution still allowing complete haemolysis was taken to be the titre

2) The beta haemolysin titre was determined as above with the following modifications All dilutions were made in phosphate buffered saline containing 0 001 M Mg SO_4 Sheep erythrocytes were used and during the second hour the samples stood in a refrigerator at +4° C.

3) Delta haemolysin was measured in the same way as alpha haemolysin but [illegible] atants were oping serum was an antitoxin to crude toxin preparation of the staphylococcus strain 196 E, which is the international prototype for the production of enterotoxin A This strain also gives a high yield of α haemolysin The immunization procedures have been described in an earlier paper (5)

Disintegration of bacteria was performed in a X press according to Edebo (1)

RESULTS

Initial Phenomenon for the Selection of Strains

In the routine sensitivity test for antibiotics a rise of haemolytic activity was observed as circular zones round the discs of some antibiotics at the border of growth of the staphylococcus strains examined As appears from Fig 1 this was true for benzylpenicillin as well as for methicillin ampicillin and to some extent for chloramphenicol The additional antibiotics in the test did not produce this phenomenon The haemolytic area for benzylpenicillin corresponded to a concentration of the antibiotic of 0 02–0 3 IU/ml

Haemolytic Activity after Growth in Liquid Medium Containing Falling Concentrations of Benzylpenicillin and Methicillin

Both antibiotics stimulated in sub bacteriostatic concentrations the production of staphylococcus haemolysin It is seen from Table 1 that

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MATERIAL AND METHODS

Strains of *Staphylococcus aureus* were selected from the daily routine examinations Twenty strains showing the typical haemolytic zone were chosen of which ten were resistant to benzylpenicillin and ten sensitive For each of these one strain with the corresponding sensitivity but without a haemolytic zone was isolated For the gel diffusion test a strain (13671) was used which was sensitive to penicillin but which showed no haemolytic zone

Antibiotics

Benzylpenicillin sodium (Penicillinsodium Astra Sweden lyophilized in lots of 1 000 000 IU) and methicillin sodium or 2 6 dimethoxiphenyl penicillin sodium (Belfacillin Astra Sweden lyophilized in lots of 1 gr) were used in the main experiments and in some tests ampicillin chloramphenicol and some other antibiotics

The two first mentioned antibiotics were stored in lots at —20° C diluted in saline to 100 IU/ml and 10µgr/ml Each lot was thawed and used in the dilution tests only once

Antibiotic Sensitivity Test

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3) Delta haemolysin was measured in the same way as alpha haemolysin but human erythrocytes type 0 Rh + were used instead of rabbit ones

Lipase was determined as described in a previous paper (4)

Gel diffusion was performed as described in a previous paper (5) Supernatants from cultures of the strain 13671 in falling concentrations of benzylpenicillin were concentrated five times according to Kohn (6) and used as antigens Developing serum was an antitoxin to crude toxin preparation of the staphylococcus strain 196 F which is the international prototype for the production of enterotoxin A This strain also gives a high yield of α haemolysin The immunization procedures have been described in an earlier paper (5)

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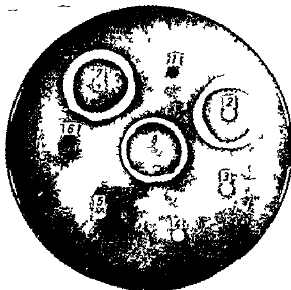


Fig 1

Disc diffusion plates with haemolytic zones round the penicillin discs
1 sulfa 2 benzylpenicillin 3 erythromycin 4 streptomycin 5 tetracyclin 6 chloromycetin 7 ampicillin 8 methicillin

strains not showing a haemolytic zone on sheep blood agar plates produced haemolysin in cultures containing concentrations of methicillin between 2.5 0.009 μgr/ml with a peak at 0.75 μgr per ml. There was a statistically significant difference in titres between this culture and the control culture which did not contain methicillin. There was a trend in the same direction for strains showing haemolysis on sheep blood agar plates but less pronounced.

TABLE 1

Reverse ahaemolysin titres at Different Concentrations of Methicillin

Staphylococcus aureus	No of strains	Methicillin μgr per ml 5	2.5	1.25	0.75	0.37	0.18	0.009	0
Strains with a haemolytic zone	10	0	3.6	54.4	67.4	60.0	52.8	52.0	52.0
Strains without a haemolytic zone	10	0	3.2	22.8	26.4	19.2	12.0	10.0	2.8

The haemolysin titres and growth curves in three typical strains without haemolysis on sheep blood agar plates are shown in Fig. 2.

Corresponding to the experiments with methicillin on the penicillin resistant strains the sensitive strains also showed the same trend in haemolysin production tested against benzylpenicillin as is seen in Table 2.

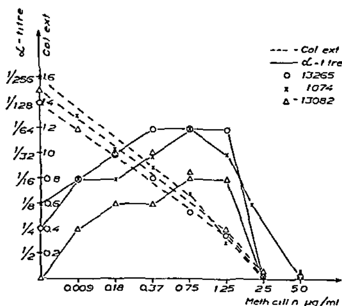


Fig 2

Alpha haemolysin titres for three strains at different concentrations of methicillin Broken lines indicate growth curve

TABLE 2

Reverse a haemolysintitres at Different Concentrations of Ben ylpenicillin

Staphylococcus aureus	No of strains	Benzylpenicillin (IU per ml) 0 13	0 08	0 06	0 04	0 03	0 02	0 015	0
Strains with a haemolytic zone (average titres)	10	2 4	4 0	5 6	10 4	32 4	50 0	38 4	4 0
Strains without a haemolytic zone (average titres)	9+1*	0	0 4	0 8	6 4	9.2	22 8	36 8	13.2
Strain 13671	1	0	0	4	32	64	64	128	8

* Strain 13671

As appears from the table, 0 02 0 015 IU per ml of benzylpenicillin turned out to be the optimal concentration for the haemolysin production

Gel Diffusion Test

As appears in Fig 3 at least three precipitation lines occurred for the cultures containing benzylpenicillin in concentrations between 0 12 and 0 015 IU per ml The middle line of the three was clearly seen for the concentration of 0 08 IU per ml and was equally visible in all lower

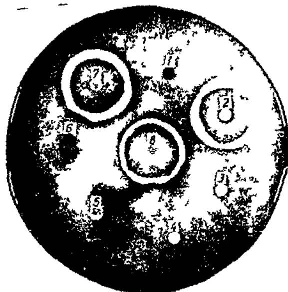


Fig 1

Disc diffusion plates with haemolytic zones round the penicillin discs *1* sulfa *2* benzylpenicillin *3* erythromycin *4* streptomycin *5* tetracyclin *6* chloromycetin *7* ampicillin *8* methicillin

strains not showing a haemolytic zone on sheep blood agar plates produced haemolysin in cultures containing concentrations of methicillin between 2 5 0 009 µgr/ml with a peak at 0 75 µgr per ml There was a statistically significant difference in titres between this culture and the control culture which did not contain methicillin There was a trend in the same direction for strains showing haemolysis on sheep blood agar plates but less pronounced

TABLE 1

Reverse ahaemolysintitres at Different Concentrations of Methicillin

Staphylococcus aureus	No of strains	Methicillin µgr per ml							
		5	2 5	1 25	0 75	0 37	0 18	0 009	0
Strains with a haemolytic zone	10	0	3 6	54 4	62 4	60 0	59 8	57 0	52 0
Strains without a haemolytic zone	10	0	3 2	23 8	26 4	19 2	12 0	10 0	2 8

The haemolysin titres and growth curves in three typical strains without haemolysis on sheep blood agar plates are shown in Fig 2

Corresponding to the experiments with methicillin on the penicillin resistant strains the sensitive strains also showed the same trend in haemolysin production tested against benzylpenicillin as is seen in Table 2

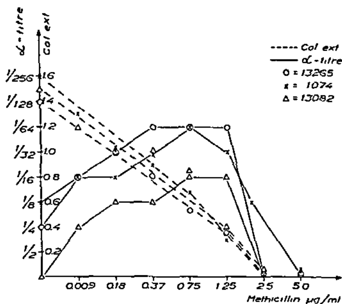


Fig 2

Alpha haemolysin titres for three strains at different concentrations of methicillin Broken lines indicate growth curve

TABLE 2

Reverse α-haemolysintitres at Different Concentrations of Benzylpenicillin

Staphylococcus aureus	No of strains	Benzylpenicillin (IU per ml) 0.13	0.08	0.06	0.04	0.03	0.02	0.015	0
Strains with a haemolytic zone (average titres)	10	2.4	4.0	5.6	10.4	32.4	50.0	38.4	4.0
Strains without a haemolytic zone (average titres)	9+1*	0	0.4	0.8	6.4	9.2	22.8	36.8	13.2
Strain 13671	1	0	0	4	32	64	64	128	8

* Strain 13671

As appears from the table, 0.02–0.015 IU per ml of benzylpenicillin turned out to be the optimal concentration for the haemolysin production

Gel Diffusion Test

As appears in Fig. 3 at least three precipitation lines occurred for the cultures containing benzylpenicillin in concentrations between 0.12 and 0.015 IU per ml The middle line of the three was clearly seen for the concentration of 0.08 IU per ml and was equally visible in all lower

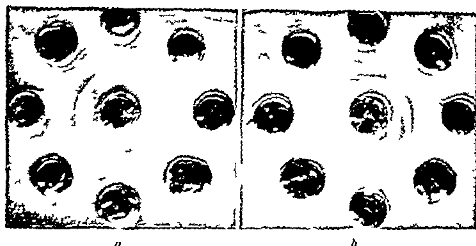


Fig 3

Gel diffusion of supernatants of cultures from the *staphylococcus* strain 13071 containing falling concentrations of penicillin G developed by an anti[illegible] (Strain 196 F) in the central well Peripheral wells from the top to the right a) 0.32 0.25 0.16 0.12 0.08 0.06 0.04 0.03 IU/ml b) 0.02 0.015 0 IU/ml The last five saline

concentrations but not for the culture exempt of penicillin and not at *higher concentrations of penicillin The fairly broad line nearest to the* antigen wells also showed some extra precipitation patterns for the cultures with low contents of penicillin

Effect of Disintegration

The supernatants after centrifugations of the staphylococci which has been disintegrated in the X press contained neither haemolysin nor lipase

Effect of Penicillin on the Production of other Extracellular Products

Throughout the work the production of lipase was studied in addition to the haemolysin studies Not all strains produced lipase but of those who did none was stimulated to produce it in excess by penicillin as is shown in Table 3 and 4

TABLE 3

Reverse Lipasetitres in Cultures of Penicillin Resistant Staphylococcus Strains at Different Concentrations of Methicillin

	No of strain	Conc of Methicillin (µgr per ml)						
		2.5	1.25	0.75	0.37	0.18	0.009	0
Lipase (average titres)	11	0	0.9	1.8	2.7	2.7	2.9	4.2

TABLE 4

Reverse Lipasetitres in Cultures of Penicillin Sensitive Staphylococcus at Different Concentrations of Benzylpenicillin

	No of strain	Conc of benzylpenicillin (IU per ml)				
		0.04	0.03	0.02	0.015	0
Lipase (average titres)	14	0	0.1	0.9	1.9	4.1

Five strains were also studied with regard to haemolytic activity against human (delta haemolysin) and sheep erythrocytes (β haemolysin). An illustrative experiment is given in Table 5. The results suggested a similar stimulation of delta and beta haemolysin though to a lesser extent than that of alpha haemolysin. However, a crossreaction of alpha haemolysin on human and sheep blood could not be excluded.

TABLE 5

Reverse Titres of Haemolytic Activity Against Different Kinds of Erythrocytes in the Presence of Benzyl Penicillin Strain 1325

Benzylpenicillin (IU/ml)	2.5	1.25	0.75	0.36	0.18	0.09	0
rabbit erythrocytes	0	64	64	64	32	16	4
sheep erythrocytes	0	16	8	4	0	0	0
human erythrocytes	0	8	0	0	0	4	4

DISCUSSION

Evidence of a stimulating effect on the production of staphylococcus haemolysin by penicillin preparations has been presented in this report. An increased release of antigens in the presence of penicillins was confirmed by gel diffusion analysis.

A paradoxical stimulation of staphylococcal growth registrated by viable count has been reported in several papers. There is no evidence, however, that the phenomenon described in this report could be caused by a general stimulation of bacterial activity as it appeared when bacterial growth was strongly suppressed.

Neither could it be due to a simple burst of bacteria after weakening of the cell wall by interaction of penicillins as the phenomenon could not be reproduced on lipaseproduction. In the same direction points the fact that no haemolysin activity could be found after disintegration of the staphylococci in the X press according to *Edebo*.

The gel diffusion test gives evidence that some extra antigens were produced in cultures containing low concentrations of penicillin and especially the middle line in this test was exclusively found in cultures supposed to be stimulated by penicillin. The active haemolysin acted

chiefly on rabbit red cells indicating the presence of alpha haemolysin. Concerning the stimulating effect on haemolysis on human and sheep blood it was uncertain whether this was due to a real release of delta and beta haemolysins respectively or to a crossreaction of alpha haemolysin. Unpublished data (*Hallander & Bengtsson*) however, indicate that such crossreactions will occur only when there is a high titre of alpha haemolysin. The observation however, that haemolysin for human blood is produced may be of some interest. *In vivo* an intermuscular injection of 300000IU of benzylpenicillin will give a concentration in the patients blood of 0.2 IU per ml or more for three hours. After four hours, however, this concentration has fallen to 0.03 IU per ml or very close to concentrations which *in vitro* activate the haemolysin formation.

SUMMARY

Some strains of *Staphylococcus aureus* caused in the antibiotic sensitivity test on sheep blood agar plates a fairly narrow zone of haemolysis round the penicillin discs at the border of beginning bacterial growth. It was also shown that a significantly higher production of staphylococcus haemolysin occurred in cultures containing small amounts of benzyl penicillin or methicillin in comparison with cultures without the addition of penicillin preparations.

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IMMUNOCHEMICAL STUDIES ON ANTIGEN PREPARATIONS FROM STAPHYLOCOCCUS AUREUS

3 *The n Antigen*

By

ARNE GROV, BERIT MYKLESTAD and PER OEDING

Received 3 ii 66

The *Staphylococcus aureus* antigen *n* was demonstrated by *Haukenes & Oeding* (9) in 1960 It agglutinates on slides and also gives a characteristic precipitation line in agar gel with *n* factor serum (9, 7) The *n* antigen was found to be heat labile, to be destroyed by trypsin and was suggested to be of protein nature Of 19 type strains, 5 contain the antigen strains 1503, 2253, 28, 3647, and Cowan III

The present study was undertaken to isolate and to characterize more fully the chemical and serological nature of the *n* antigen

MATERIALS AND METHODS

Strain Staph aureus strain 1503 (14) was chosen because it seems to be rich in *n* antigen Its serological pattern is *e m n* and the phage pattern *52 52A/80 81/82/7/42F/54/73/83*

Growth and harvesting 18 hrs cultures of the strain on nutrient agar in Petri dishes of 14 cm diameter at 37° C, were harvested by scraping with a glass rod

Ion exchange columns of diethyl amino ethyl (DEAE) cellulose (Eastman) 100 230 mesh and carboxy methyl (CM) cellulose (Sigma Chemical Company) and *columns for gel filtration* of Sephadex G 25 G 75 G 100 and G 200 (Pharmacia Uppsala Sweden) were employed

Crystalline trypsin was obtained from Novo Copenhagen and digestions were performed at pH 7 2 (0 2 M phosphate buffer) and 37° C (0 1 per cent soln enzyme/substrate ratio 1/100 by wt)

Dialysis was carried out in cellophane tubes (Kalle & Co Wiesbaden) against running tap water for three days and finally against distilled water for one day

Hydrolysis was performed in sealed nitrogen filled tubes in 3 N hydrochloric acid for 3 hrs at 100° C and in 6 N hydrochloric acid for 18 hrs at 105° C

The hydrolysates were [illegible]

•

H O (70 15 15) (17)

As spray reagents a) aniline hydrogenphthalate in water saturated butanol (16) b) sodiumperiodate benzidine (2) c) ninhydrin (5) d) Elson Morgan reagent (15) and e) isatin (11) were employed

The qualitative *Molisch test* for carbohydrates was carried out as described in (8)

chiefly on rabbit red cells indicating the presence of alpha haemolysin Concerning the stimulating effect on haemolysis on human and sheep blood it was uncertain whether this was due to a real release of delta and beta haemolysins respectively or to a crossreaction of alpha haemolysin Unpublished data (*Hallander & Bengtsson*), however, indicate that such crossreactions will occur only when there is a high titre of alpha haemolysin The observation however, that haemolysin for human blood is produced may be of some interest *In vivo* an intermuscular injection of 300000IU of benzylpenicillin will give a concentration in the patients blood of 0 2 IU per ml or more for three hours After four hours, however, this concentration has fallen to 0 03 IU per ml or very close to concentrations which *in vitro* activate the haemolysin formation

SUMMARY

Some strains of *Staphylococcus aureus* caused in the antibiotic sensitivity test on sheep blood agar plates a fairly narrow zone of haemolysis round the penicillin discs at the border of beginning bacterial growth It was also shown that a significantly higher production of staphylococcus haemolysin occurred in cultures containing small amounts of benzyl penicillin or methicillin in comparison with cultures without the addition of penicillin preparations

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Isolation Experiments

The alcohol precipitate from the phenol phase was only soluble in phenolic solution and in strong alkali However successive washings of the precipitate with buffer removed protein A together with some of the *n* antigen After several washings no further release of either protein A or *n* antigen from the precipitate could be observed A suspension of the washed precipitate in saline still gave a strong *n* line in agar whereas no other precipitation line could be seen

Collected washings containing protein A and *n* antigen were concentrated under reduced pressure dialysed placed on a DEAE cellulose column and eluted with a continuous increase in an ammonium formate (pH 6 8) gradient according to the procedure described in (6) The fractions were examined spectrophotometrically in a Unicam SP 500 spectrophotometer at 280 mμ by ring test precipitation and by agar gel precipitation All serologically active material was liberated in the range of 0 3 0 6 M ammonium formate No separation of the two antigens was obtained Most of the ultraviolet light absorbing material went straight through the column but some also followed the active material Serologically positive fractions were collected concentrated *in vacuo* dialysed and fractionated once more on an identical column The active material still contained both antigens in the whole region but the ultraviolet light absorption was markedly reduced The serologically active material was desalted by dialysis and freeze-dried This material is referred to as *preparation 1*

Further experiments to separate the two antigens in preparation 1 were carried out with various buffers on DEAE cellulose columns on CM cellulose columns and on columns of Sephadex gel of various pore size but they were all unsuccessful

Since it seemed impossible to regain the *n* antigen from the alcohol precipitate of the phenol phase by washing or extraction whereas it had been shown to diffuse from a suspension of the precipitate into the gel the following method was tried Five ml of a 2 per cent agar gel (Difco Noble) was transferred to a 10 ml beaker Saline suspension of the precipitate freed from protein A by washing was poured into a central basin 6 mm in diameter The beaker was closed and incubated for 24 hrs at 37° C With a corkbore (8 mm in diameter) closing the central basin the gel outside was removed from the beaker suspended in 4 ml of saline and centrifuged (30 mins at 18 000 RPM) Two further extractions of the gel were carried out with 2 ml portions of saline The collected eluates were filtered through a fine grade filter paper dialysed and freeze dried The material obtained was a white amorphous and very light material soluble in distilled water and giving a sharp n line on agar precipitation No other precipitation lines could be observed This material is referred to as *preparation 2*

Owing to shortage of material no further purification experiments

have been tried up to the present, and the following chemical and serological experiments have been carried out with the two impure preparations

Chemical Properties

Contrary to protein A, which was only faintly positive, preparation 1 gave a strong Molisch reaction showing the presence of carbohydrates Preparation 2 as well as an extract of pure agar gel were positive in the Molisch test

3 N hydrochloric acid hydrolysates were examined chromatographically for aldohexoses and aldopentoses in solvent systems A and B Spray reagent a) revealed bands corresponding to authentic samples of galactose, glucose, mannose, and xylose Galactose seems to be the major sugar component, but traces of glucose are present A 3 N hydrochloric acid hydrolysate of extract from pure agar gel showed galactose when examined chromatographically

Examinations for sugar alcohols were carried out chromatographically with solvent system C and spray reagent b) A band moving identically with that of a glycerol standard was observed with 3 N hydrolysates of both preparations No bands corresponding to hexosamines could be observed using solvent system A and spray reagent d)

In the 6 N hydrochloric acid hydrolysate of preparation 2 seven amino acids were detected chromatographically using spray reagents c) and e) The amino acids lysine, glutamic acid, glycine, alanine, proline, valine, and leucine, showed movements identical with those of authentic specimens in solvent systems A, D, and E Only traces of a few amino acids could be observed in the hydrolysate of a pure agar gel extract

Serological Properties

Preparation 2 showed a single line on agar gel precipitation corresponding to the *n*-line given by crushed 1503 bacteria As a control in the agar precipitation test, serum 3189 was placed in the basin next to serum 1503 because the *n* line is the only one not common to these two systems The *n*-line was still intact after heating to 70° C but had disappeared after heating to 100° C Tryptic digestion of the preparation showed no effect upon the precipitating activity, whereas periodate treatment destroyed it

In the haemagglutination test, preparation 2 showed no sensitizing ability either of normal or of tanned sheep erythrocytes

The results of the serological experiments carried out with preparation 1 are shown in Tables 1 and 2 In addition to the *n* antigen this preparation also contains protein A After tryptic digestion only the *n* line could be seen on agar diffusion and after periodate treatment only the protein A line Absorption of serum 1503 with purified protein

A removed the antibodies against this antigen, whereas the antibodies producing the *n*-line were intact When serum 1503 was absorbed with trypsin digested preparation 1, the antibodies responsible for the *n*-line were removed while the antibodies producing the protein A line were intact When the serum was absorbed with periodate treated preparation, the opposite was observed These results strongly indicate that the component responsible for the *n*-line on precipitation is of carbohydrate nature

TABLE 1

Agar Precipitations

Antigens	1503 Serum	Serum 1503 absorbed with			
		Prot A	Prep 1	Prep 1 digested w trypsin	Prep 1 treated w $NaIO_4$
1503 bacteria	Poly A Prot A *n*	Poly A *n*	Poly A	Poly A Prot A	Poly A *n*
Prep 1	Prot A *n*	 *n*	–	Prot A	 *n*
Prep 1 digested w trypsin	*n*	*n*	–	–	*n*
Prep 1 treated w $NaIO_4$	Prot A	–	–	Prot A	–

indicates no line in the systems

TABLE 2

Agglutinations on Slides

Antigen	*n factor* serum unabsorbed	*n* factor serum absorbed with		
		Prep 1	Prep 1 digested w trypsin	Prep 1 treated w $NaIO_4$
1503 bacteria	++		++	-

indicates no agglutination

The agglutination experiments with *n* factor serum (Table 2) show that absorption of serum with untreated and periodate treated preparations completely inhibits the agglutination reaction, while absorption with trypsin-digested preparation does not This should indicate a component of protein nature responsible for the agglutination in *n* factor serum

DISCUSSION

In contrast to most of the antigens previously isolated from *Staph aureus* strains, extraction of the *n* antigen was surprisingly difficult Beside the chemical composition this difficulty may also be related to the function and location of the antigen in the bacteria

It seems likely that the *n* antigen is quite firmly attached to other components in the bacteria, *e g* protein A, and can be released only in contact with soluble compounds or solids It could not be released from the alcohol precipitate by washing, but diffused easily into agar

The preparations described in the present study are impure However, the results of chemical as well as of serological examinations strongly indicate that the *n* antigen is composed of two entities a carbohydrate component and a polypeptide component

Glycerol and identical sugars were found in both preparations Traces of glucose and xylose have earlier been demonstrated in hydrolysate of protein A (6) but as no protein A could be demonstrated in preparation 2 by the sensitive serological test, glucose and xylose seem either to belong to the *n* antigen or they are contaminants Galactose was demonstrated in the hydrolysate of extract of pure agar gel, but galactose was the major sugar component in preparation 1 and is not present in protein A

Though traces of a few amino acids could be demonstrated in the hydrolyzed extract of pure agar gel, the intensity of the amino acid bands revealed on chromatograms of hydrolysed preparation 2, clearly showed that these amino acids originate from material that has diffused into the gel With the exception of aspartic acid and serine, preparation 2 contains the same amino acids that were found in protein A A certain similarity in physical properties rather than a real linkage may possibly be the reason why we did not succeed in separating the two antigens in preparation 1 chromatographically

Since the *n* antigen is found in the phenol phase after extraction and also is precipitated by hydrochloric acid (8), it is likely that the polypeptide is the major component

The suggestion that the observed sugars and amino acids belong to the *n* antigen is supported by the serological experiments Tryptic digestion had no influence on the precipitating ability whereas periodate treatment destroyed it These results were evidenced by absorption of serum with trypsin and periodate treated preparations followed by diffusion in agar (Table 1) and indicate that the precipitating ability is due to the carbohydrate component

n factor serum has previously been shown to give agglutination and to produce the *n* line on agar diffusion against bacteria containing the *n* antigen (9) The present agglutination experiments showed complete absorption of agglutinins from *n* factor serum with untreated and periodate treated preparations while trypsin digested preparations did

not absorb the agglutinins (Table 2) This shows that the agglutinating ability of the *n* antigen is due to a component of protein nature

The *n* antigen has been defined by its activities the *n* factor in agglutination and the specific precipitation line in agar It now appears that these activities are due to different chemical components a polypeptide and a carbohydrate component respectively The components must be quite firmly attached to each other and probably constitute one molecular entity To give a complete chemical characterization of the *n* antigen and of its active groupings, it is necessary to prepare a purer material in greater amounts

SUMMARY

Purified preparations of the *n* antigen have been isolated from phenol extracts of crushed *Staph aureus* 1503 bacteria Chemical examination indicates that the *n* antigen is composed of two components a carbohydrate and a polypeptide

Paper chromatographic examination of hydrolysates showed glycerol, galactose, glucose (trace), mannose, and xylose These amino acids were detected lysine, glutamic acid, glycine, alanine, proline, valine, and leucine

The results of serological experiments, in connection with trypsin and periodate treatment of the preparations and absorption of sera, show that the precipitating and agglutinating abilities of the *n* antigen are most probably due to the carbohydrate and polypeptide components, respectively

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SERRATIA MARCESCENS INFECTIONS IN PREMATURE INFANTS

By

A Stenderup, Ole Færgeman and Mogens Ingerslev

Received 25 ii 66

Serratia marcescens is usually regarded as a species with low pathogenicity in man Nevertheless infections caused by this micro organism seem to occur with increasing frequency It may cause septicaemia as well as infections of the urinary and respiratory tracts and in other localities Sometimes infections may be acquired in hospitals and spread among the patients In diagnosing these infections, attention must be paid to the fact that strains of Serratia that do not produce detectable pigment occur more commonly than those that do so (*von Graevenitz* 1964, *Ewing et al* 1962)

From February 1964–June 1965 inclusive we observed in the Maternity Hospital for Jutland, Aarhus, 13 cases of infections in the newborn, in which acromogenic Serratia marcescens played the sole or a major rôle as the aetiological agent

OWN SERIES

All 13 cases but one occurred in premature babies 11 boys and 2 girls Six babies (birth weight from 1160–1760 g (average 1570 g)) died from septicaemia In one case septicaemia was suspected but the child recovered In six cases purulent conjunctivitis was the only sign of infection

In all cases of septicaemia the clinical picture was fairly uniform The temperature was 2 degrees or less below 37° C which is not unusual in prematures The babies were weak, periodically grey with attacks of respiratory distress and cyanosis In five cases localized erythema developed in the face in the neck or on the legs All deaths occurred within 2 11 days (average 6 days) after births

The autopsy findings were also fairly uniform showing partial atelectasis and congestion of the lungs and occasionally bleedings in the alveoli cerebral oedema and stasis organorum In two cases bleedings were found in the cerebral ventricles and three cases revealed signs of meningitis

In cultures from the organs (usually the spleen, liver, lung and sometimes the brain and the heart), *Serratia marcescens* was the only organism isolated in two cases In three it was the predominant organism, but other gram negative rods (*Escherichia* coli, *Pseudomonas*

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aeruginosa or *Klebsiella*) were also cultured In one case both *Serratia marcescens* and *Pseudomonas aeruginosa* were found in abundance

In the baby who recovered from suspected septicaemia arthritis developed in the left knee joint Erythema was also noted The diagnosis was based on the presence of *Serratia marcescens* in cultures from the nose and the umbilicus

All six cases with conjunctivitis were diagnosed by means of cultures of eye secretions In four cases Serratia marcescens was the only organism found in two it was the predominant organism but occurred together with *Staphylococcus albus* *B anitratum* or *Pseudomonas aeruginosa* The infections lasted from one to several weeks but all ran a benign course

BACTERIOLOGY

According to *Lautrop* the genus *Serratia* must agree with the definition of the family *Enterobacteriaceae* ferment glucose producing 2 3 Butylene glycol form coiling peritrichic flagella liquefy gelatine rapidly but must not ferment rhamnose *Serratia marcescens* is further characterized by not forming acid in arabinose xylose and raffinose Lysine and ornithine are decarboxylized All our strains fitted this description but none of them formed detectable pigments The cultural behaviour which was alike in all the strains is shown in Table 1

TABLE 1
Cultural Behaviour of Serratia marcescens

Adonitol	+	Glycerol	(+)
Dulcitol	—	Malonate	
Sorbitol	+	Indole	
Arabinose		H S	—
Xylose		Gelatine	+
Rhamnose		Ammonium glucose	+
Maltose	+	Ammonium citrate	+
Salicin	(+	KCN	+
Inositol	+	KNO_3	+
Lactose		Voges Proskauer	+
Sucrose	+	Urea	
Mannitol	+	Arginine	
Glucose	+	Lysine	+
Gas production	*very scanty*	*Ornithine*	+
Raffinose	—	Pigment	
Trehalose	+	Motility	+
Sorbose		Oxidase	
Inulin			

The growth of three different strains was tested at 30 34 and 38° C The optimum was at 38° C although there was very little difference between the three temperature levels

Sensitivity tests on solid medium revealed resistance to penicillin and ampicillin partial resistance to tetracyclins and novobiocin and sensitivity to kanamycin neomycin and streptomycin

EPIDEMIOLOGY

In all cases, the babies were nursed in the premature unit and in all but two cases they had been kept in incubators A common source of Serratia marcescens was not found in the environment A total of 126 cultures were made from the incubators, various utensils, walls taps etc As a selective medium rhamnose plates containing 1 unit ml of penicillin were chosen Only colonies not fermenting rhamnose were selected for identification Only 10 of the samples were positive for Serratia marcescens, and abundant growth was never observed

Other sources might be the vagina and the intestinal contents of the mothers, but material from these sources was not subjected to bacteriological study

To obtain information as to how often newborn infants harbour *Serratia marcescens* without having overt infections, all 15 babies (9 boys and 6 girls) in the premature unit were examined on a randomly selected day Cultures were made of material from the oral cavity and from the anal region

In six babies (5 boys and 1 girl) *Serratia marcescens* was isolated from the oral cavity, and in two boys also from the anal region

One strain from each patient and all strains isolated from the environment and from healthy babies were typed by Dr *W H Ewing*, Department of Health, Education, and Welfare, Atlanta, Georgia, U S A

They all belonged to O group 14, H 12 except one strain which was reported as O group 14 H undetermined

DISCUSSION

It is generally assumed that impaired general condition, prolonged treatment with antibiotics and or antimitotics, concurrent infections caused by other organisms and anatomical abnormalities (*e g* of the urinary or respiratory tract) are factors which predispose to infections caused by organisms of low virulence, such as *Seratia marcescens* Premature infants must be assumed to have a decreased resistance to infections In all our cases, penicillin had been administered routinely from birth and was not withdrawn until infection with gram negative bacteria was suspected, after which treatment was started in accordance with the results of sensitivity tests

In some cases, other gram negative organisms were isolated together with *Serratia marcescens*, in these, it is difficult to assess their mutual role as aetiological agents Nevertheless, our cases show that various species of gramnegative bacteria and sometimes *Serratia marcescens* alone, may be a serious threat to premature babies, especially boys It seems beyond doubt that a low-grade epidemic was rife in the Maternity Hospital By now our investigations have unfortunately not revealed the source of these infections which occurred regularly in spite

of a high hygienic standard in the unit The degree and frequency with which especially aeromogenic *Serratia marcescens* participates in the normal intestinal and genital flora in women are unknown and may be fruitful subject of investigation in an attempt to disclose the origin of aeromogenic *Serratia marcescens*

SUMMARY

Thirteen cases of infections in premature infants (11 boys and 2 girls) are reported In some cases, *Serratia marcescens* was the only aetiological agent Six out of seven patients died from septicaemia In six cases, purulent conjunctivitis was the only sign of infection The source of infection was not found All strains isolated were aeromogenic, showed similar biochemical reactions and were of the same serotype

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GINGIVAL CHANGES IN DIABETES MELLITUS

1 Vascular Changes

By

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Received 30 ii 66

Recent publications on diabetes mellitus have dealt in large measure with the vascular changes which manifest themselves in patients who have been diabetic for many years Frequently these vascular changes predominate the syndrome and influence the prognosis but their pathogenesis has not yet been elucidated

Kimmelstiel & Wilson (1936) were the first to discover in the kidneys of long standing diabetics a peculiar lesion to which they gave the name intercapillary glomerulosclerosis Later *Friedenwald* (1950) concluded that the vascular lesion which results in the retinopathy is specifically related to the diabetes that the renal lesion described by Kimmelstiel and Wilson is a very closely related condition *Lundbæk* (1949) advanced the hypothesis that all vascular anomalies in diabetics were due to a generalized specific diabetic angiopathy *diabetic angiopathy Burger* (1954) who shared this view pointed out the lack of histological and histochemical studies of the capillary network in diabetics In recent years this hypothesis has received support from many workers (*Fagerberg* 1959 *Balslev Jorgensen* 1962 *Bloodworth* 1962 *Ditzel* 1962) and vascular changes have been demonstrated in a large number of different organs and tissues in diabetic patients (*McManus* 1950 *Ashton* 1953 *Burstein et al* 1957 *Aagenæs & Haagensen* 1958 *Fagerberg* 1959 *Goldenberg et al* 1959 *Angervall et al* 1961 *Balslev Jorgensen* 1961 1962 *Aagenæs* 1961 *Ditzel* 1962 *Bloodworth* 1963 *Pedersen & Olsen* 1962 *Camerini Davalos et al* 1963 *Garde & Kugelberg* (1963) *Reske Nielsen & Lundbæk* 1963 *Riedel* 1965) These changes are characterized by the periendothelial deposition of a periodic acid Schiff (PAS) positive diastase resistant material in many capillaries and larger vessels

Only a few histological studies of the gingiva in human diabetics are on record In 5 diabetics ranging in age from 18 to 51 years and with a history of diabetes of from 6 months to 5 years *Gescheff* (1931) found inflammatory infiltrations in the subepithelial layers which ex

Aided by a grant from the *Foundation for the support of dental research*

hibited ample depositions of lipids *Ziskin, Loughlin & Siegel* (1944) have reported histological studies of the gingiva from 14 juvenile and 5 adult diabetics, all treated with insulin, and from 2 non-insulin-treated adult diabetics The duration of diabetes was from 4 months to 15 years In the insulin-treated cases they found hyperkeratinization and hyperplasia of epithelium and of connective tissue as well as prominence of the capillary bed In the non-insulin-treated diabetics they found hyperkeratinization *Ray* (1948), studying 30 diabetics from 10 to 71 years of age with a duration of diabetes from 3 months to 10 years, found thickening and hyalinization of the walls of large and small blood vessels in 14 cases He had no control series *Ray & Orban* (1950), moreover, investigated 30 diabetics in the age range 25 to 71 years with a duration of diabetes of from 3 months to 10 years as well as 6 juvenile diabetics from 4 to 12 years of age for whom the duration is not stated They could not confirm the findings described by previous authors and concluded that no changes characteristic of diabetes could be found in the gingiva *Stahl, Witkin & Scopp* (1962) investigated 95 male in-patients aged 20 to 71 years Several had diabetes mellitus and/or hypertensive cardiovascular disease The duration of the disease is not stated These authors found degenerative arteriolar changes in the majority of gingival specimens obtained from patients with known diabetes mellitus and/or hypertensive cardiovascular disease In gingival biopsies from 3 pre-diabetics (prediabetes means "before diabetes mellitus", i e from conception to the first abnormal glucose tolerance test From a genetic point of view, a person with identified diabetic parents is probably prediabetic at birth, but he may have, for many years, an entirely normal glucose tolerance test), aged 7 to 44 years, *Camerini et al* (1963) found no abnormalities with the light microscope Most recently, *McMullen, Gottsegen, Legg & Camerini-Davalos* (1965) studied 20 patients, overt chemical (overt diabetics have clinical symptoms and abnormal blood sugar and chemical diabetics have no clinical symptoms, but abnormal glucose tolerance test), and prediabetics as well as normal controls Of these patients five overt, five chemical, and five prediabetics showed vascular abnormality of the small vessels of the alveolar mucosa when studied with the light microscope Vascular morphology was normal in control patients as was vascular morphology in attached gingiva in all the subjects

The present investigation was undertaken because the question concerning the presence of possible vascular changes in the marginal gingiva has not yet been clarified, and because sufficient regard has not previously been paid to the patients' ages and the duration of their disease The objects were to ascertain

1) whether young patients with diabetes mellitus show vascular changes in the marginal gingiva,
2) the nature of such possible vascular changes,

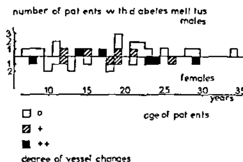


Fig 1

Age and sex distribution of patients with diabetes mellitus of less *than 10 years duration*

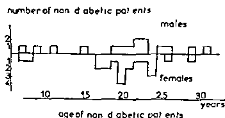


Fig 2

Age and sex distribution of the non diabetic patients

3) their incidence,
4) and how early such possible vascular changes may be diagnosed histologically with the light microscope

This investigation is the first of a series dealing with pathological manifestations, histological as well as clinical, in the oral cavity of patients with diabetes mellitus

MATERIAL

The patients were selected according to the following criteria

1) [illegible] ge in order to rule out changes [illegible]
2) [illegible]
3) [illegible] to have set in before the age of 30 In that case it is easier to state the accurate duration of the disease
4) The clinically known duration had to be less than 10 years
5) Pregnancy must have been excluded
   The control patients also had to comply with items 1 and 5 Moreover
6) None of the control persons must suffer from any known systemic disease
7) There must not be a family history of diabetes mellitus Thereby the occurrence of prediabetics and latent diabetes was limited

The material comprised 37 patients with diabetes mellitus 16 females and 21 males who attended out patient follow up in the Steno Memorial Hospital All were being treated with insulin and had normal bl[illegible]d pressure There were no cases of retinopathy nephropathy or neuropathy The average age was 17[9]/[12] years,

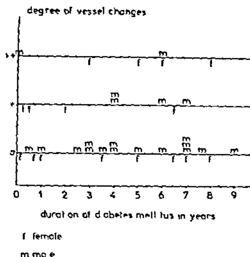


Fig 3

Degree of gingival vascular changes in patients with diabetes mellitus in relation to the duration of the disease (assessed clinically)

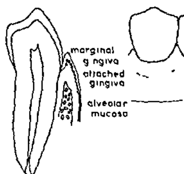


Fig 4

Topographic classification of the gingiva

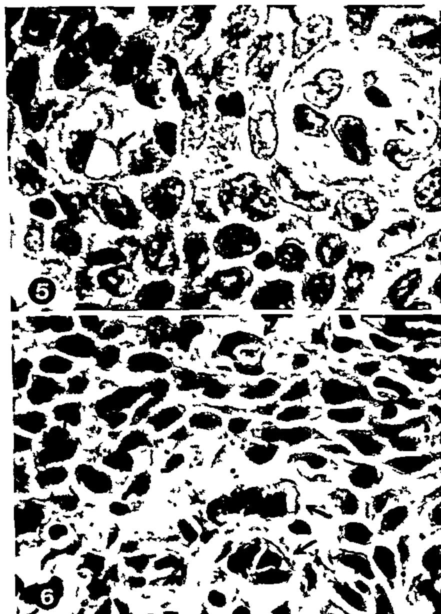
range 7 to 34 years (cf Fig 1) The average duration of diabetes was 4 4/12 years (Fig 3)

The control group consisted of 35 healthy patients from a private dental practice The 15 males and 20 females selected had an average age of 20 years, range 7 to 31 years (cf Fig 2)

As nicotine influences the blood vessels and epithelium the proportion of smokers was kept identical in both groups

METHOD

The clinical odontological examination was performed from 10 12 a m in order to avoid possible hour to hour fluctuations in the diameters of the vessels Under local block analgesia using Carbocain® Dental 3 per cent an analgesic without adrenaline a biopsy specimen was removed from the lower canine and premolar region, in that area of the buccal marginal gingiva (cf Fig 4) which was least inflamed The biopsies were fixed in neutral buffered formalin embedded in paraffin in the usual way and cut into sections of 5 6 μ stained with haematoxylin eosin and periodic acid Schiff (PAS) (*Balslev Jorgensen* 1961) with an without



Figs 5–6

Fig 5 Photomicrograph of normal capillaries from a cross sectioned connective tissue papilla from the marginal gingiva of a 29 year old non diabetic patient PAS stained Magnification 1333 ×

Fig 6 Photomicrography of capillaries from a cross sectioned connective tissue papilla from the marginal gingiva of a 1[illegible] year old patient with diabetes mellitus of 4 years duration PAS stained Magnification 1333 ×

diastase (Merck 0.1 per cent). The PAS staining of all preparations from diabetics and controls, was carried out at the same time, in the same solutions.

Vascular changes of the connective tissue, if any, were assessed according to the following semi-quantitative evaluation of PAS stained preparations:

0 Vessels without changes

+ Mild to moderate PAS positive mural thickening in a few vessels

++ Moderate PAS positive mural thickening in many vessels or pronounced thickening in a few

+++ Very pronounced PAS positive mural thickening

RESULTS

In 14 out of 37 diabetics there were vascular changes in the marginal gingiva (cf. Fig. 6), while such changes could not be demonstrated in the control group (Fig. 5). The result prove to be statistically significant, $P < 0.1$ per cent, using the χ^2 test with Yate's correction.

The vascular changes were particularly striking in PAS-stained preparations in which PAS-positive, diastase-resistant thickenings of the vessel walls were seen especially in the capillaries. These changes were unevenly distributed in the same patient, the same preparation exhibiting normal as well as affected vessels. The endothelial cells were often swollen, and a few arterioles showed proliferation of endothelial cells. In several cases the vascular lumina were obliterated. The basement membrane between the epithelium and connective tissue was often thickened as compared with the findings in non-diabetics.

Haematoxylin-eosin stained preparations from diabetics showed in some cases hyalinization of the vessel walls and often obliterated lumina. In several cases there were intercellular changes with splitting of the collagen fibres. A more detailed investigation of the basement membrane between the epithelium and connective tissue as well as of the intercellular substance is going on.

The vascular changes were found as early as two weeks after diabetes mellitus had been diagnosed.

DISCUSSION

The histological changes in the vessels of the marginal gingiva of diabetics in the present study are identical with the vascular changes found by others in cases of late diabetic complications in other organs and tissues, such as the kidneys, retina, joints, lower limbs, stria vascularis of the inner ear, etc. Thus, the findings support the view that diabetics are affected by systemic diabetic angiopathy. From Fig. 3 it may be seen that the distribution of patients with diabetes mellitus having vascular changes is even throughout the duration of diabetes. However, the present series is too small to form the basis of an assessment as to whether these vascular changes increase with increased duration of the disease. In this connection, it should be mentioned that the study comprised only patients with clinically recognized dia-

betes mellitus of less than 10 years duration and that none had retinopathy or nephopathy The early occurrence of vascular changes is not surprising when considering that vascular changes have been detected in prediabetics (*Ditzel* 1954 *McMullen et al* 1965) and that *Burger* (1954) has observed early vascular changes of the retina in newly discovered elderly diabetics The vascular changes found in the present study are not influenced by the blood pressure or other systemic diseases and must be purely diabetogenic

Presumably the PAS positive substance prevents a normal function of the vessel as seen in the skin (*Aagenaes & Moe* 1961) This may reduce the exchange of chemical substances between blood and tissue, tissue and blood thus diminishing the possibilities of defense and regeneration

Further information concerning the chemical nature of the PAS positive deposits cannot be obtained from the present study

SUMMARY

Gingival biopsies from 37 diabetic females and males with a duration of clinically manifest disease of less than 10 years and of an average age of 17½ years were compared with corresponding biopsies from 35 healthy females and males of an average age of 20 years The histological specimens were stained with haematoxylin eosin and periodic acid Schiff (PAS)

Vascular changes of the marginal gingiva were found in 14 of the 37 diabetics

The vascular changes may be found when the diagnosis of diabetes mellitus is established

No such changes were observed in the control patients

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THE AMYLOID NATURE OF THE HOMOGENEOUS SUBSTANCE IN THE CALCIFYING EPITHELIAL ODONTOGENIC TUMOUR

By

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Received 26 iii 66

The calcifying epithelial odontogenic tumour, first described by *Pindborg* in 1955 and 1958 is now recognized as a destinctive entity among the epithelial odontogenic tumours The tumour originates in most cases from the reduced enamel organ (probably from the stratum intermedium) but an origin from the epithelium of the oral mucosa has also been suggested (*Pindborg* 1966)

A recent analysis of 26 cases (*Pindborg* 1966) has shown no sex predilection but a preference (65 per cent) for the mandible The tumour is most often associated with an embedded tooth usually in the premolar molar region The radiographic picture is one of translucency interspersed with small radiopacities

Histologically the tumour consists of sheets of polyhedral cells in a connective tissue stroma The polyhedral epithelial cells tend to be closely packed but are occasionally split up in islands or strands by a hyalinized stroma The cell borders are distinct and the cytoplasm slightly eosinophilic The nuclei vary much in shape and size but mitotic figures are rarely seen The cytoplasm has a tendency to undergo degeneration and become homogeneous The degenerated cytoplasm has affinity for mineral salts and calcification occurs in the form of Liesegang's rings Also the surrounding collagenous tissue may become mineralized

A histochemical study (*Gon* 1965) has shown that the tumour cells are comparable to the cells making up the stratum intermedium of the enamel organ suggesting an origin from the structure This view was put forward by one of the authors in 1958 and later supported by *Chaudhry et al* (1962)

The purpose of the present paper has been to study the possible amyloid nature of the homogeneous substance in cases of the calcifying

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epithelial odontogenic tumour utilizing several staining techniques for demonstrating amyloid During the accomplishment of the present investigations a similar study was reported by *Vickers et al* (1965)

MATERIAL AND METHODS

The material comprised tissues from calcifying epithelial odontogenic tumours from 7 patients five of which derive from the Royal Dental College of Copenhagen the rest being submitted by Dr Francis Howell La Jolla California

All tissues employed were obtained by biopsy or by surgical removal In most cases specimens from two or three different sites in the same individual tumour were employed After formalin fixation and paraffin embedding sections were cut

amyloid was identified by its Congo red positivity and by its birefringence when viewed under crossed polars In addition its specific fluorescence with thioflavin T in the U V light was used

RESULTS

Varying amounts of homogeneous substance were found in all specimens investigated It was invariably found to fulfil the criteria of amyloid material, i e it showed birefringence with Congo red and specific fluorescence with thioflavin T

The localization and the morphology of the amyloid substance seemed to vary with the age or the differentiation of the tumour In seemingly "young" tumours with large sheets of epithelial cells and very sparse or absent calcification practically all of the amyloid was found as small, round or ovoid deposits scattered among the epithelial tumour cells (Figs 1, 3 and 4) Very often flattened nuclei or nuclear debris were found in the periphery of the small deposits (Fig 3) Frequently small amounts of homogeneous substance could be recognized within the cytoplasm of the epithelial cells (Fig 1), thus indicating a cellular origin from the neoplastic cells In tumours showing more abundant stroma with scattering of the epithelial sheets a coales

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Figs 1-6

Fig 1 Fluorescent amyloid deposits in sheets of neoplastic epithelial cells Note occasional intracellular fluorescence Thioflavin T combined phase contrast and U V light × 400

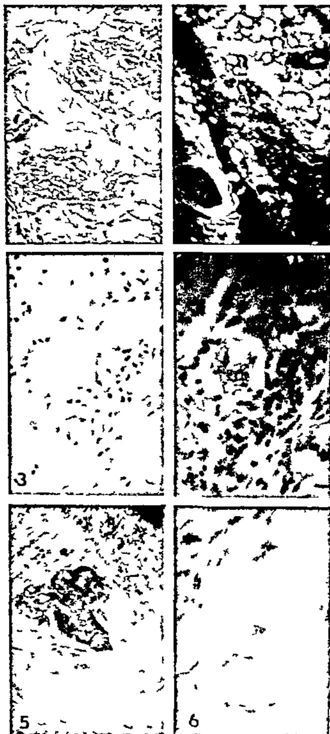
Fig 2 Isolated small amyloid deposits within the epithelial sheets and coalescence of the amyloid in the stroma and in the periphery of a small vessel Thioflavin T U V light × 100

Fig 3 Small rounded amyloid deposits with flattened nuclei and nuclear *debris in* their periphery Note central incipient calcification Alkaline Congo red × 400

Fig 4 Same field of vision as in Fig 3 Alkaline Congo red crossed polars × 400

Fig 5 Calcified area containing remnants of amyloid deposits Alkaline Congo red crossed polars × 100

Fig 6 P A S positive cells in the stromal amyloid Periodic acid Schiff × 400


1
3
5
6

cence of the amyloid bodies was evident thus presenting a picture of a more diffuse stromal amyloidosis resembling the distribution usually found in systemic amyloidosis (Fig. 2). In the larger pools of amyloid incipient calcification was noted in many instances often as darker greyish spots in the central portion (Fig. 3). Within the larger calcified areas amyloid could constantly be recognized (Fig. 5). An interesting feature was the many strongly P.A.S. positive cells in the immediate vicinity of the amyloid deposits (Fig. 6).

DISCUSSION

In the present study the amyloid nature of the homogeneous substance in the calcifying epithelial odontogenic tumour has been established in all the specimens investigated. The appearance of the amyloid substance in this rare tumour corresponded closely to that of ordinary systemic amyloidosis; its structure was finely fibrillar, it stained pink with Congo red and yielded a brilliant green birefringence under crossed polars. With thioflavin T it showed the characteristic fluorescence in the U.V. light. Methyl violet or toluidine blue stains were not employed in our investigations, but in the report by *Vickers et al.* (1965) metachromasia with crystal violet was demonstrated.

Local amyloid formation in malignant tumours of various kinds has been described occasionally in the literature for many years (*Sumegi et al.* 1951). Until recently interest has been focused mainly on the local occurrence of amyloid within plasmocytomas (*Dahlin & Dockerty* 1950). In 1959 *Hazard et al.* described the characteristics of the so called medullary carcinoma of the thyroid gland. The common features of these tumours were a circumscribed solid appearance, cellular variations of round, polyhedral and spindle cells in various architectural patterns of pseudopapillary, follicular and festoon like arrangements and above all the consistent finding of amyloid in the stroma. These findings have later been confirmed in large materials by others (*Freeman & Lindsay* 1965, *Albores Saavedra et al.* 1964). This tumour is hitherto the only type of malignant tumour in which the local occurrence of amyloid substance is considered a necessary diagnostic feature.

In the light of *Teilum's* observations during the last two decades a cellular morphogenesis was to be expected (*Teilum* 1964). In our observations and in those of *Gon* (1965) and those of *Vickers et al.* (1965) the intracellular origin of the amyloid deposits seems unequivocal. In this connection the observations on the medullary thyroid carcinoma by *Albores Saavedra et al.* (1964) are of special interest. They were able to demonstrate directly the formation of stromal amyloid from neoplastic thyroid cells in tissue culture.

The relation between the amyloid and the calcified areas of the calcifying epithelial odontogenic tumour is not quite clear. In the present material certain features indicate a deposition of mineral salts

in the preformed amyloid substance Among these the observation that calcification is usually preceded by a coalescence of the small amyloid deposits that small calcified areas may be encountered in the central portions of amyloid deposits and finally that small remnants of amyloid substance regularly are found scattered within the calcified areas Calcification of amyloid substance was already described in 1917 by *Glaus*

From the present investigations it may be concluded that the demonstration of amyloid substance is probably a necessary diagnostic feature of the calcifying epithelial odontogenic tumour In addition it seems justified to regard the amyloid substance in this tumour as a matrix for a subsequent calcification

SUMMARY

In a histologic investigation of 7 cases of the calcifying epithelial odontogenic tumour the characteristic homogeneous substance was found in all cases investigated to fulfil the criteria of amyloid material That is positivity and birefringence with Congo red and specific fluorescence with thioflavin T

Evidence is presented which supports the assumption of an origin from the neoplastic cells of the amyloid material in this particular tumour In addition it seems probable that the amyloid serves as a matrix for the subsequent calcification It is suggested that the demonstration of amyloid is considered a necessary diagnostic feature of the calcifying epithelial odontogenic tumour

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STUDIES IN ORAL LEUKOPLAKIAS

10 *Periodic Acid Schiff Staining of Normal and Leukoplakic Epithelium before and after Vitamin A Application*

By

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Received 13 v 66

The glycogen content in various areas of the oral cavity have been studied (*Wislocki et al* 1951 *Weinmann et al* 1959 *Weiss et al* 1959 *Meyer & Gerson* 1964) It can be concluded from these reports that there is an inverse correlation between the glycogen content in the oral epithelium and the degree of keratinization *Haim* (1964) and *Bulow* (1966) have described the glycogen content of the normal buc cal mucosa Haim found that non keratinized oral mucosa did not con tain glycogen whereas the keratinized mucosa has a high content Contrary to this Bulow found abundant glycogen in the non kerati nized buccal mucosa Investigations have also been made of the rela tion between subepithelial inflammation and glycogen content in the gingival epithelium (*Turesky et al* 1959 *Weiss et al* 1959 *Weinmann et al* 1959 *Meyer & Medak* 1962) The conclusion of these reports is that the amount of glycogen in gingival epithelium is in direct ratio to subepithelial inflammation In epithelium from alveolar mucosa no correlation could be found between glycogen content and inflamma tion

Only a few studies have dealt with the content and distribution of glycogen in the epithelium of oral leukoplakias (*Fasske & Themann* 1959 *Turesky et al* 1961 *Vachrameeva* 1964) The results of these reports are not in agreement *Fasske & Themann* found glycogen in the epithelium from oral leukoplakias but only in the middle and upper part of stratum spinosum The stratum basale the lower part of stratum spinosum and the keratinized surface layers were free of glycogen *Vachrameeva* stated that the amount of glycogen decreases when the keratinization increases However in the hyperplastic zones

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of the epithelium in her verrucous type of leukoplakias the amount of glycogen was strongly increased *Turesky et al* found that glycogen rarely occurred in the orthokeratotic epithelium of leukoplakias In the parakeratotic epithelium glycogen occured in stratum spinosum and in the surface layers Also, they stated that the occurence of glycogen was related to the degree of epithelial hyperplasia rather than to the nature of the surface changes

Diastase resistant periodic-acid Schiff staining material (d r -material) was demonstrated in various degrees in different areas of the oral epithelium by *Wislocki et al* (1951) The d r -material was found in the intercellular spaces or at the cell surfaces in the outer part of the epithelium, either as granules or as a homogeneous substance *Turesky et al* (1959) found in the epithelium of oral leukoplakias the d r -material to stain more deeply in the parakeratotic layers than in the orthokeratotic layers They concluded, that there is an inverse relationship between the stainability of this material and the degree of keratinization

The purpose of this investigation was to study 1) the content and distribution of glycogen and d r -material in the epithelium of clinical normal oral mucosa and of oral leukoplakias, 2) whether inflammation has any influence on the glycogen-keratinization ratio for oral leukoplakias and 3) the effect of vitamin A upon the correlation between the content of glycogen, d r -material, and the type of keratinization

DEFINITIONS

Leukoplakia is for the purpose of this investigation selected according to the following criterias any white patch or plaque more than 5 mm in diameter on the oral mucous membrane that 1) cannot be removed by scraping 2) cannot be reversed by removing obvious irritants and 3) cannot be classified clinically or microscopically as another diagnosable disease (*Silverman et al* 1963 a b *Renstrup et al* 1965)

Hyperorthokeratosis is a pathologic condition in which the superficial layers of the epithelium are keratinized containing no nuclei and appearing homogenous and strongly acidophilic In areas where orthokeratosis normally occurs it has to exceed the thickness of that of the control area to be called hyperorthokeratosis (*Renstrup et al* 1965)

Hyperparakeratosis is a pathologic condition in which the outer cell layers are flattened containing pyknotic nuclei and exhibiting strong acidophilia In areas where parakeratosis normally occurs it has to exceed the thickness of that of the control area to be called hyperkeratosis A parakeratosis or a hyperparakeratosis can be *with* or *without* a stratum granulosum (*Renstrup et al* 1965)

Inflammation To evaluate the degree of infiltration with lymphocytes and plasmacells in the biopsy material in this study four graduations were used 1) no inflammation 2) slight inflammation 3) moderate inflammation and 4) heavy inflammation

MATERIAL AND METHODS

The same fifteen patients with oral leukoplakia as used in a previous study of phosphatases (*Renstrup et al* 1965) were studied The age varied from 37 to 74 years and the sex distribution was 10 men and 5 women The leukoplakias were localized to the cheek and commissural area in 11 cases and to the inferior surface of the tongue in 4 cases

Vitamin A was given as described previously (*Silverman et al* 1963 a b) as 75 000 unit vitamin A acetate troches Ten troches per day for 2 to 3 weeks were prescribed and the patients were instructed to place a troche until dissolved against the lesion to be studied

Biopsies were taken from the leukoplakic lesions and from the adjacent normal mucosa of all patients before as well as after vitamin A application

Under local infiltration anaesthesia (lipocain)—care was taken not to inject directly into the site of biopsy—five millimeter punch biopsies were taken The tissue was fixed in neutral formalin embedded in paraffin and sectioned at 6μ

Glycogen and d r material were demonstrated by the method of *McManus* (1961) Demonstration of d r-material was performed with diastase (Maltase Merck) 0 1 per cent in 0 02 M phosphate buffer pH 6 0 for 1 hour at 37° C prior to the periodic acid Schiff reaction

As control served sections where the oxydation with periodic acid was omitted they were all negative

Hematoxylin and eosin staining of sections of all biopsies was performed and served to determine the type of keratinization and the degree of inflammation

RESULTS

The epithelium from the control area showed either no keratinization of the surface or a slight parakeratinization The leukoplakic lesions could be divided histologically into three groups based upon the keratinization type of the epithelium 1) hyperorthokeratosis 2) hyperparakeratosis *with* a stratum granulosum and 3) hyperparakeratosis *without* a stratum granulosum

Glycogen and d r material The *hyperorthokeratotic* epithelium from the leukoplakias did not show staining reaction in any layer, either for glycogen or for d r material In the leukoplakias demonstrating *hyperparakeratotic* epithelium *with* a stratum granulosum glycogen was found in a few cases as few and scattered granules in the cytoplasm of cells in the upper part of stratum spinosum In all cases a d r material was seen in the intercellular spaces in the upper part of stratum spinosum and in the parakeratotic surface layers The epithelium from leukoplakias characterized by *hyperparakeratotic* epithelium *without* a stratum granulosum showed glycogen as granules in the cytoplasm of the cells in the middle and upper part of stratum spinosum and in some of the parakeratotic surface cells In the upper part of stratum spinosum and in the parakeratotic surface a d r material was found in the cell borders or in the intercellular spaces

The distribution of glycogen was the same in the parakeratotic epithelium as in the unkeratinized epithelium from clinically normal mucosa All the cells in the middle and upper part of the stratum spinosum and in the surface layers showed glycogen granules in the cytoplasm The intensity of the staining decreased towards the basal part of the stratum spinosum where few scattered granules were seen Stratum basale was free of glycogen The d r material was found at the cell borders or in the intercellular spaces in the middle and upper part of stratum spinosum and in the surface layers The basement membrane contained d r material in all cases

In six cases of leukoplakia the vitamin A application changed the

keratinization type of the epithelium from hyperorthokeratosis or hyperparakeratosis *with* stratum granulosum to a hyperparakeratosis *without* stratum granulosum or to an unkeratinized epithelium In all six cases the glycogen content had increased in the epithelium after the vitamin A administration

In the remaining nine cases no change was found in the type of keratinization, but in seven cases the thickness of the keratinized layers was reduced The glycogen content in the epithelium after vitamin A application had not changed in any of the nine cases

The vitamin A administration did not change the keratinization type for the clinically normal oral mucosa, neither was the amount and distribution of glycogen and d r -material influenced by vitamin A

The local vitamin A application had no direct influence on the content and distribution of glycogen and d r -material in the epithelium of oral leukoplakias and normal oral mucosa But, in the cases where vitamin A altered the keratinization type of a leukoplakia from a higher *to a lower degree of keratinization the glycogen content increased accordingly*

The degree of inflammation showed a decrease after vitamin A administration in seven cases of leukoplakia, in six cases there was no change, and in the remaining two cases there was a slight increase in inflammation In the clinically normal oral mucosa no inflammation was found neither before nor after vitamin A application No correlation could be found between degree of inflammation and the content of glycogen and d r -material in the epithelium

DISCUSSION

The distribution of glycogen in the oral epithelium as it is found in the present study is in agreement with other investigations (*Fasske & Themann* 1959, *Weiss et al* 1959, *Turesky et al* 1961) The glycogen is located to the upper part of stratum spinosum, while the basal cell layers of the epithelium are free of glycogen

The relation between glycogen content and keratinization type of the epithelium, as it is found here, is in agreement with other investigations (*Fasske & Themann* 1959 *Turesky et al* 1961, and *Yachrameeva* 1964) if the keratinization types of the epithelium in oral leukoplakias can be considered as different degrees of keratinization Thus, the hyperorthokeratosis could be considered as a higher degree of keratinization than hyperparakeratosis *with* a stratum granulosum, and this type again as a higher degree than hyperparakeratosis *without a stratum granulosum* There is no glycogen in the epithelium with a high degree of keratinization (hyperorthokeratosis) while an increasing amount is found in epithelia with decreasing degrees of keratinization This inverse relationship between content of glycogen and degree of keratinization in the epithelium is not specific for oral leukoplakias

as it is found in normal oral epithelia from different areas with different degrees of keratinization (*Turesky et al* 1959, *Weinmann et al* 1959, *Weiss et al* 1959, *Meyer & Medak* 1962)

The present investigations shows no correlation between the glycogen content in the epithelium of oral leukoplakias and the degree of inflammation This finding is in agreement with that of *Weinmann et al* for alveolar mucosa, while other authors have found a direct relation between subepithelial inflammation and glycogen content for gingival epithelium (*Turesky et al* 1959, *Weiss, Weinmann & Meyer* 1959, *Meyer & Medak* 1962)

The d r-material first described by *Wislocki et al* is also found in the present study in both leukoplakic and normal epithelium except hyperorthokeratotic epithelium The findings of *Turesky et al*, *Weinmann et al*, and *Meyer & Gerson* that the stainability of the d r-material is increased as the surface is approached could not be confirmed in this investigation, whereas the inverse relationship between stainability of d r-material and the degree of keratinization, reported by *Turesky et al* (1959, 1961) and by *Weinmann et al* (1959), agreed with the present study

An attempt to explain the difference in glycogen content in epithelium with a high degree of keratinization and with a low degree of keratinization has been made The precursor to glycogen, probably glucose 6 phosphate, is supposed to participate in the process of keratinization The absence of glycogen in the basal layers of epithelium might indicate that only the precursor to glycogen is present here Highly keratinized epithelium may utilize the carbohydrates in the basal part of the epithelium, while unkeratinized epithelium may convert the surplus of carbohydrates to glycogen

SUMMARY

The occurence and distribution of glycogen and diastase resistant periodic acid Schiff staining material (d r-material) in the epithelium of leukoplakic lesions and normal oral mucosa have been studied The results have been correlated to type of keratinization, degree of inflammation and to local application of vitamin A It was found that glycogen, when present, is localized in the upper part of stratum spinosum as granules in the cytoplasm An inverse relationship between glycogen content and degree of keratinization could be demonstrated This relationship is not influenced by vitamin A No correlation between glycogen content and degree of inflammation could be found The d r material was found at the cell borders of the superficial layers of nonkeratinized and parakeratinized epithelium, whereas it is absent in orthokeratinized epithelium

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SINUSLYMPHOCYTOSIS AND LYMPH FLOW

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Peripheral lymph contains very few cells, but receives a great number of small lymphocytes during its passage through the lymph nodes Therefore the intermediate and central lymph which enters the blood flow is very rich in cells (*Yoffey* 1960) *Gowans* (1959) has demonstrated that a great number of these small lymphocytes have recirculated to the lymph nodes from the blood by passing through the walls of the postcapillary veins, located to the inner layer of the cortex The lymphocytes subsequently pass into the sinus system and hence back to the blood stream This new knowledge is important for the interpretation of some of the variations seen in the histologic picture of the lymph nodes, for instance the lymphatic hyperplasia and sinus lymphocytosis, which will be discussed in the present communication

The diffuse lymphatic hyperplasia is characterized by a marked increase of small lymphocytes in the parenchyma and sinus system of the lymph node The sinus lymphocytosis may sometimes be so intense that the density of small lymphocytes may be higher in the sinus than in the surrounding parenchyma (*Lennert* 1961) The mechanism behind these "reactive' changes is not clear The object of the present investigation has been to see if sinus lymphocytosis may result from alterations in the lymphocyte recirculation, for instance by retardation of the lymphocyte output via the efferent lymph This situation may occur if the lymph flow through the node is blocked or greatly slowed down

METHODS

Our experiments have been performed on the right knee node in hooded rats The knee node receives only peripheral lymph which contains very few cells The lymphocytes in the node therefore arrive from the recirculating pool, via the post capillary veins and by mitotic activity inside the node The left knee node has been used as a control

Three different methods have been utilized to reduce the lymph flow through the lymph nodes

1) Resection of the afferent lymph vessel under Nembutal ether anesthesia The lymph node was exposed by splitting the muscle on the medial side of the fossa poplitea Thereafter a small amount of Patent Blue Violet (PBV) (*Kinmonth* 1954) was injected between the first and second toe and the extremity moved back and forth until the dye was visible in the node and in the efferent lymph vessels All the fat tissue around the distal pole of the lymph node and in the caudal part of the

fossa poplitea was thereafter removed together with the afferent lymph vessels New PBV from the depot in the foot was then mobilized to check that all afferent vessels had been resected Finally the wound was sutured and covered with Nobecutan

2) Ligation of the afferent lymph vessel The node was exposed as described After injection of PBV the lymph vessels were ligated with silk no 6 0 Thereafter PBV again was mobilized to make sure that all afferent vessels were ligated The wound was then closed

3) Immobilization of the extremity *White et al* (1933) have demonstrated that complete immobilization of the limb reduces the lymph flow to near zero The animal was placed on its back and anesthezised with Nembutal for 2 hours Initially both hind limbs were moved for one minute Thereafter the right hind limb was fixed in a relaxed position by means of a tape over the nails The left hind limb remained free and was exercised for thirty seconds every five minutes during the observation period in order to keep the lymph flow going on on that side

The animals were sacrificed with ether The knee nodes were gently removed fixed in Carnoy's solution, serially sectioned and stained in haematoxylin and eosin

RESULTS

Series 1· Resection of the Afferent Lymph Vessels

Thirty rats were randomized into seven groups which were sacrificed 5 hours, 1, 2, 3, 4, 7 or 10 days after the afferent lymph vessels were resected At sacrifice it was checked by injection of PBV in the drainage area if any lymph vessels has been overlooked during the operation, or if new vessels had been organized in the period between the operation and sacrifice

During the first 24 hours after operation, free, blue coloured lymph was observed in the wound cavity However, it was found that up to the fourth day after resection, lymph flow to the knee node was blocked, and no PBV could be seen in the knee node on the operated side Seven and ten days postoperatively the lymph flow was partly or completely reestablished in all animals, as all knee nodes were stained blue, though in half the animals only traces of colour could be seen All non-operated limbs had blue knee nodes

Microscopic examination revealed that 5 hours postoperatively the right and left knee nodes were practically identical No accumulation of small lymphocytes was found in the lymph nodes on the operated side

Twenty-four hours postoperatively a severe accumulation of small lymphocytes was found in the marginal and intermediate sinuses in the lymph nodes on the operated side, while the nodes on the left side appeared normal But for the difference in the lymphocyte accumulation the node on both sides had the same histologic appearance

The second and third day postoperatively the histologic picture was approximately the same, but the difference between the operated and non-operated sides was possibly more marked at this time (Fig 1 and Fig 2) In some operated nodes the parenchyma and frequently also the efferent lymph vessels (Fig 3) were densely packed with small lymphocytes

At later times, the sinuses in the operated nodes still contained more



Fig 1

Right knee node 48 hours after resection of afferent lymph vessels Intermediate sinus is full of small lymphocytes H + F ca 200 ×



Fig 2

Left knee node from the same rat as seen in Fig 1 The cell population in the sinuses is normal Most of the cells are reticulum cells and mast cells There are only a few lymphocytes H + F ca 200 ×

lymphocytes than did the controls but the difference in cell content was less marked At these intervals it was also noticed that animals which showed only traces of PBA in the nodes had more lymphocytes in the sinus than the animals which had strongly coloured lymph nodes From the second day on a slightly increased vascularization and some fewer granulated mast cells were found in some of the operated nodes



Fig 3

Right knee node 48 hour's after resection of afferent lymph vessels Part of the cortex with a germinal center and an efferent lymph vessel packed with small lymphocytes H + E ca 200 ×



Fig 4

Right knee node 48 hours after ligation of afferent lymph vessels Marginal sinus and intermediate sinus are packed with small lymphocytes H + E ca 200 ×

Series 2 Ligation of the Afferent Lymph Vessels

In this experiment 4 animals were used, and all were sacrificed 24 hours after the operation The PBV test was performed as above and indicated that in two animals the ligature blocked the lymph flow *completely* *In the two other animals lymph nodes and efferent lymph*

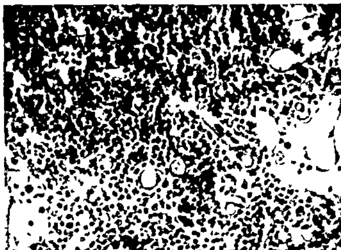


Fig 5

Right knee node 48 hours after insufficient ligation of afferent lymph vessels Few cells in marginal and intermediate sinus H + E ca 200 ×

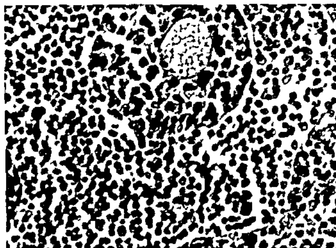


Fig 6

Right knee node two hours after immobilization under anaesthesia The medullary cords are seen in the center and in the periphery The intermediate sinus is full of lymphocytes H + E ca 400 ×

vessels were coloured, and the dye must have passed through the ligature In the animals where the ligature was efficient a histologic picture was found which was practically identical with the findings 24 hours after resection of afferent lymph vessels (Fig 4) In animals where the dye had passed through the ligature we did not observe any significant accumulation of lymphocytes in the node (Fig 5)

Series 3 Immobilization of the Right Hind Limb

In this experiment two animals were used They were sacrificed after 2 hours' immobilization In both animals we found a marked accumulation of small lymphocytes in the intermediate sinus of the knee node of the immobilized side (Fig 6), but is was not so abundant as in the resection and ligation series In particular there were fewer lymphocytes in the hilus area and in the efferent lymph vessels In the control nodes from the left side, the lymphocyte population was normal

DISCUSSION

After resection of afferent lymph vessels the knee nodes show a distinct sinus lymphocytosis The changes are very marked 24 hours postoperatively and remain so as long as the lymph flow through the node is blocked or strongly reduced These results thus seem to confirm the assumption that the sinus lymphocytosis may be caused by impaired lymph flow, resulting in delayed evacuation of lymphocytes via the efferent lymph vessels The correlation with the lymph flow was also clearly demonstrated in the second series where the lymphocytosis failed to appear in animals with ineffective ligature of the afferent lymph vessels In the two first experiments, however, it could not be excluded that the operative trauma or PBV or a combination of both could account for the lymph node reaction We therefore performed the immobilization experiment where this parameter could be omitted Also in this experiment a marked sinus lymphocytosis was observed The reaction was only slightly less than seen in the first two series, although the leg was immobilized for two hours only (The operative trauma is supposed to be the reason why lymphocytosis was not found five hours after resection of the afferent vessels)

The sinuses in the experimental lymph nodes were as wide as those in the controls and the absolute number of lymphocytes was higher Thus, the sinus lymphocytosis can not be caused by collapse of the sinus system and must indicate genuine accumulation of lymphocytes within the node

As practically all the cells which accumulate in the sinuses during two hours' reduced lymph flow are small lymphocytes, there is a strong indication that these cells belong to the recirculating pool and do not arise from cell division within the node If so, the immobilization method could be used to estimate the number of recirculating cells within a single node These problems are under investigation

The correlation between the reduced lymph flow and sinus lymphocytosis, as found in the present experiments, may account for the great variability in lymphocyte content often observed in normal lymph nodes It also could explain the findings in some pathologic conditions such as diffuse lymphatic hyperplasia which is observed especially in mesenterial lymphadenitis in children, in hernia lymph nodes, and in

lymph nodes regional to malignant tumours (*Lennert* 1961) In malignant tumours it appears possible that lymphocytosis may be a result of impaired lymph flow through the nodes as a consequence of blocked lymph vessels, in hernia lymph nodes also as a result of immobilization of the extremity in connection with surgery The cause of mesenterial lymphadenitis in children is not known *Lennert* (1961) points out that the lymphocytosis hardly can be caused by invasion of cells from the pulpa and follicles He mentions the possibility that there may be some connection with the lymphocyte recirculation which will be in accordance with the present results

In the present experiments it has been observed that even a brief immobilization caused a marked accumulation of lymphocytes in the sinuses of the nodes It can be expected that exercise will bring these cells into the blood stream in the course of a few minutes A similar mechanism could be responsible for the early observations of a decrease of white cells in peripheral blood after some time of rest and the relative lymphocytosis immediately after exercise (*Rous* 1908, *Sturgis & Bethell* 1943)

SUMMARY

The relationship between the lymphocyte content of the sinuses in the knee node and the lymph flow through the nodes has been investigated in rats Blockage of the lymph flow through the node by ligation or resection of the afferent lymph vessels caused a marked sinus lymphocytosis the first days postoperatively The same changes were observed —although to a smaller degree—when the lymph flow through the nodet was reduced by immobilization of the extremity for two hours Practically all lymphocytes in the sinuses were small lymphocytes which indicates that they belong to the recirculating pool

It is concluded that the sinus lymphocytosis which can be observed in different conditions in man, may be caused by blockage of the lymph flow to the node It is further proposed that the sinus lymphocytosis due to reduced lymph flow may account for the leucocyte fall in peripheral blood during rest and a lymphocytosis immediately after exercise It is suggested that the immobilization method may be used to estimate the number of recirculating lymphocytes in a single node

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HODGKIN'S DISEASE AND KAPOSI S SARCOMA

Report of a Case

By

NARVE MOE

Received 2? ix 66

Kaposi s sarcoma or idiopathic multiple hemorrhagic sarcoma is a disease of the skin consisting of an eruption of multiple bluish red or dark brown nodules and plaques which may undergo ulceration The sites of predilection are the distal parts of the extremities Visceral lesions occur in about 10 per cent of the cases and the most frequent sites are the gastro-intestinal tract the liver the lungs and the retro peritoneal and the mesenteric lymph nodes (*Lever* 1961)

The coincidence of Kaposi s sarcoma and malignant lymphoma is not an uncommon occurrence *Allen* (1954) reported a series of 41 cases of Kaposi s sarcoma and 17 per cent of the patients also had a malignant lymphoma In another series of 24 cases 2 of the patients had a co existent malignant lymphoma (*Moertel & Hagedorn* 1957) *Abrahamsen & Welleland* (1959) reported a case with the coexistence of Kaposi s sarcoma and myelogenous leucemia

The incidence of Hodgkin s disease and Kaposi s sarcoma in the same patient is not high We have therefore found it of interest to report a case with evidence of both diseases and specially because the patient lived with the diagnosis of Hodgkin s disease from 1937 until he died of this disease in 1965 after developing Kaposi s sarcoma a few years earlier

CASE REPORT

The patient was a 69 year old man Between 1927 and 1955 he was hospitalized many times under the diagnosis of gastric ulcer which was treated conservatively In 195 [illegible] oscopy was tried [illegible] was performed [illegible] e stomach duo

From 193? the patient had noticed a tumour in the left armpit This tumour was unaltered to 1937 but at this time it increased in size He was admitted to Ullevål Hospital Oslo where an operation was performed and a group of enlarged lymph nodes was found The diagnosis after histological examination was lymphosarcoma The patient was transferred to The Radium Hospital for postoperative radiation treatment A revision of the microscopical slides was done and the diagnosis was altered to Hodgkin s disease Later controls at The Radium Hospital did not show evidence of recurrence

In 1956 the patient developed right axillary lymphadenopathy and histological examination showed a chronic lymphadenitis possibly Boeck's sarcoidosis The patient was again given radiation treatment at The Radium Hospital and by revision of the microscopical slides the diagnosis was chronic lymphadenitis However, a Hodgkin's paragranuloma could not be excluded Later controls showed no local recurrence

In 1962 oedema developed in the lower limbs and an eruption appeared in the right leg Histological examination showed Kaposi's sarcoma In november 1964 the eruption was treated by irradiation in The Radium Hospital The hemoglobin was 11 6 gm per cent, the blood sedimentation rate 75–100 mm per hour serum alkaline phosphatase 14 4 and 11 1 Bodansky units The patient complained of abdominal pains X ray examination of the lumbar column and the pelvis showed degenerative changes in the interlumbar discs, spondylitis deformans, dysplasia of the right hip joint with secondary degeneration and an ankylosis in the left hip joint

In December 1964 the patient was admitted to Ullevål Hospital with a severe hemathemesis and melena He was in shock and was treated with blood transfusions Because of continous bleeding a laparotomy was performed two days after admission and a penetrating ulcer in the duodenum was found A partial gastrectomy with gastroenteroanastomosis was done There was a small tumour in the liver and enlarged lymph nodes in the omentum and the hilar region of the liver Histological examinations of biopsies from the liver and lymph nodes showed infiltration of a malignant pleomorphous, mesenchymal tumour tissue, probably Hodgkin's disease

Seventeen days after the gastric bleeding started he was transferred to the medical department He had pulmonary oedema and stasis moderate enlargment of liver and spleen, and there was oedema of the lower limbs In the anterior part of the legs there were red blue, partly confluating infiltrations of various size The hemoglobin was 11 1 gm per cent the leucocytes count 8700 per cubic millimeter, platelets 162000 per cubic millimeter, blood sedimentation rate 33 mm per hour, icteric index 6 serum alkaline phosphatase 21 Bodansky units normal eosinophil count

After an episode of dyspnoea and tachycardia, he died on Jan the 17, 1965

PATHOLOGICAL FINDINGS

At autopsy, the patient had a chronic radiodermatitis in both axillae A small lymph node was found in the right axilla Dark blue, confluating, nodular lesions measuring up to 3 0 × 3 0 cm were present on the anterior aspects of the legs The left pleural cavity contained 1000 cc of fluid, on the right side the lung was completely *adherent The left* lung showed atelectasis, on the right large amounts of purulent secretion in the bronchi was found The heart weighed 400 gm and no significant findings was noted The stomach, duodenum, and intestines showed no signs of tumour The liver weighed 1600 gm The surface had a nodular appearance, and the cut surface showed small, confluating infiltrates The spleen weighed 480 gm, and the cut surface showed numerous grey-white infiltrates up to 2 0 × 2 0 cm Enlarged lymph nodes were present in the hilus of the liver, in the hilus of the spleen, in the mesenterium, and retroperitoneally The size was up to 2 cm in diameter, and the cut surface was greyish-white In the vertebral column numerous, white smooth infiltrates were found

Histologic sections from the eruption on the leg showed the blood vessels in the dermis dilated and increased in number They varied in size and were occasionally saccular Most of them were lined by a single layer of endothelial cells, and some were surrounded by fibroblastic cells In the stroma extravasated erythrocytes and deposits of



Figs 1–2

Fig 1 Section of dermis involved by Kaposi's sarcoma Mallory-Azan × 200
Fig 2 Section of liver with polycellular infiltrate and typical Reed Sternberg cells HE. × 200

hemosiderin were found (Fig 1) The picture was characteristic of Kaposi's sarcoma

Section from the liver, spleen and lymph nodes showed a pleomorphic, granulomatous picture with lymphocytes, plasma cells, polymorpho-

nuclear leucocytes and eosinophils, as well as many reticular cells with large nuclei and typical Reed-Sternberg cells (Fig 2) The picture corresponded to the biopsy taken by the operation two weeks earlier

DISCUSSION

The reported patient got the diagnosis of Hodgkin's disease in 1937, and was *treated with surgery and radiation* Later controls did not show evidence of recurrence until 28 years later, when symptoms of a generalized disease developed with rapid fatal outcome The disease presented itself in stage I Involvement at a single site or lymphatic region (*Peters* 1965)

Slaughter et al (1958) have treated 18 cases of Hodgkin's disease in Stage I Eleven of these (60 per cent) survived free of disease for 6-14 years Five were treated with surgery alone, and 6 had supplementary, postoperative radiation In a later report (*Slaughter* 1965) this same group of survivors had remained well with the exception of one man who died 11 years after the onset of the disease from disseminated sclerosis There was no evidence of Hodgkin's disease at autopsy Since the 1958-report, 16 more cases in the Stage I have achieved five-year-survival status, all having been treated surgically The percentage of survivors clinically free of the disease remains essentially the same as in the report from 1958

Easson & Russel (1963) have treated 64 cases of Hodgkin's disease classified as Stage I by irradiation therapy They reported a 15-year survival rate of 40 per cent In 127 cases classified as Stage III (involvement of two or more distant lymphatic regions) treated by irradiation, they found a 15-year survival rate of 11 6 per cent

Peters (1965) has treated 56 cases classified as Stage I by irradiation, and the 10-year survival rate was 48 per cent Three of the cases are 30-years survivors

None of these communications reports a case of Hodgkin's disease with such a long period of recurrence as in the case of our patient

The first case report of a coincidence of Hodgkin's disease and Kaposi's sarcoma was given by *Osborne et al* (1947) *Brunning et al* (1963) have studied the literature and found 9 cases with the coexistence of Hodgkin's disease and Kaposi's sarcoma, and they report one more case *Allen* (1954) has reported his personal experience of four cases One case is reported from Sweden (*Rajka* 1965)

Many investigators suggest that Kaposi's sarcoma is a disease of the reticuloendothelial system because of the histological picture, the occurrence of widely scattered lesions and the not uncommon occurrence of other diseases from the reticuloendothelial system in the patient (*Bluefarb* 1957)

The case here presented got the eruption of Kaposi's sarcoma 3 years before he got generalized symptoms of Hodgkin's disease

SUMMARY

A case with coexisting Hodgkin's disease and Kaposi's sarcoma is reported The diagnosis of Hodgkin's disease was made in 1937, and the patient was treated surgically with postoperative radiation Later controls did not show evidence of relapse until generalized disease and death occurred 28 years later The eruption of Kaposi's sarcoma was evident 3 years before the patient got symptoms of generalized Hodgkin's disease

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PERITONEAL EXUDATE FORMATION IN C3H MICE AND IN MICE OF AN UNRELATED CLOSED COLONY

By

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Received 2 x 66

It has previously been shown that Indian ink calls forth an inflammatory exudate on intraperitoneal injection into mice of the closed colony kept at this Institute (1) However the scatter in the amount of exudate produced is great Similarly the inflammatory response of these mice to turpentine is also characterised by great individual variation (4) If this variation were determined by genetic factors it should be possible to reduce it by using inbred mice The following experiment was set up to test this possibility

MATERIAL AND METHODS

Twelve male and 12 female C3H mice and a corresponding number of closed colony mice of similar age were used The males weighed 24 ± 1 g the females 24 ± 1 g They were each given 0 1 ml of Indian ink intraperitoneally Three days later the peritoneal exudate was removed and measured after centrifugation

Four male and 4 female C3H and closed colony mice similar to the above served as controls In these mice the fluid in the peritoneal cavity was measured in the absence of treatment

RESULTS

The findings are given in the histogram which shows the amount of peritoneal fluid present in male and female mice of both strains following the intraperitoneal injection of Indian ink, and in the absence of treatment

C3H mice In the treated mice there was no significant sex difference in the volume of fluid in the peritoneal cavity In the untreated controls there was significantly more fluid in the females than in the males ($0.01 > P > 0.001$)

Closed colony mice As in C3H mice there was no significant sex difference in the amount of fluid in the peritoneal cavity in treated mice, while the untreated females contained more fluid than the males ($0.01 > P > 0.001$)

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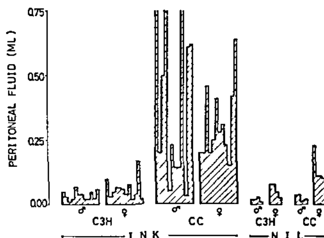


Fig 1

The volume of peritoneal fluid in C3H and closed colony mice of both sexes 3 days after the intraperitoneal injection of 0.1 ml Indian ink, and in the absence of treatment

Interstrain differences Following treatment significantly less exudate was produced by C3H mice of both sexes than by closed colony mice (0.01 > P > 0.001) The mean volumes ± S D (ml) for the C3H mice were males 0.04 ± 0.02, females 0.06 ± 0.04 and for the closed colony mice males 0.52 ± 0.49, females 0.31 ± 0.14

The differences between the untreated mice were not statistically significant

DISCUSSION

The results from the untreated mice in the present experiment confirm that the peritoneal fluid volume is greater in females than in males of our closed colony (3) and extend the observation to C3H mice No difference in the amount of fluid present in untreated mice in the two strains could be confirmed Further as has been demonstrated previously in closed colony mice (1) the sex difference disappeared following treatment in both strains

The great individual variation mentioned above in mice of our closed colony in response to an inflammatory stimulus is demonstrated in the present experiment The standard deviation from the mean in these mice was 0.49 ml for the males and 0.14 ml for the females These mice are not inbred and so variation of this kind could be due to genetic factors The variation of the response in the inbred mice used was much lower being 0.02 ml for the males and 0.04 ml for the females Thus it appears that individual variation has been cut down by using these inbred animals However if instead of considering the absolute values one looks at the variation as a percentage of the mean response the

difference is not so striking. The closed colony males gave a variation of 94 per cent and the females 45 per cent, while the C3H males gave 50 per cent and the females 33 per cent. This suggests that the individual variation may be due to phenotypic rather than genotypic factors. In consequence the use of inbred mice in an attempt to cut down individual variation in response to an inflammatory stimulus would not seem justifiable.

On the other hand the experiment also shows that there is a statistically significant difference in the intensity of the inflammatory response in these two mouse strains. This difference may well be genetically determined as the mice were matched for age and weight, and kept under identical conditions. C3H mice are "widely used in cancer research and in physiological studies, as well as in radiation and immunologic research" (5). The ability to react to an inflammatory stimulus may be a basic factor in all these fields, and the degree of the reaction may influence the outcome of the experiment. The present findings *indicate that C3H mice have little ability to respond to a non-specific irritant* that is capable of producing a copious inflammatory response in closed colony mice. As the reaction of closed colony mice does not differ markedly from that of strain A mice (2) it seems possible that it is the C3H mice that are exceptional. If this proves to be so—and *C3H mice are characterised by poor exudate production*—their use in projects in which the result is determined by minor changes in capillary permeability may be contraindicated.

SUMMARY

Exudate formation in response to the intraperitoneal injection of Indian ink was measured in C3H mice and in mice of a non-inbred closed colony. Individual variation in response was great in both strains, and is thus probably due to phenotypic variation. In contrast C3H mice produced considerably less exudate than closed colony mice, and this difference may well be genetically determined.

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KINETIC AND MEASUREMENT OF THE RENIN ANTIRENIN SYSTEM

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Received 3 vi 66

The fact that like other enzymes renin is antigenic was first shown by *Wakerlin* (8) and later confirmed by *Goldblatt* (4) and *Helmer* (7) The first two measured antirenin by its ability to neutralize the pressor effect of one dog unit renin in experiments with i v injections in dogs with a constant reaction to renin (5) *Helmer*, however, after incubation of excess renin of known concentration with the antiserum the concentration of which was to be tested has measured the unneutralized quantity of renin by the indirect method, the renin antirenin sample being incubated with angiotensinogen and the resulting angiotensin concentration being susequently biologically determined, probably by the aortastrip method

The aim of the present study has been to study 1 the kinetic of the renin antirenin system 2 the exactitude of the method of analysis in using the indirect method 3 The ability, if any, of the renin-antirenin complex to fix complement 4 The influence, if any, of the complement on this method of measuring antirenin

MATERIALS AND METHODS

The *renin* used was the commercial preparation of NBC Cleveland 28 Ohio

The *substrate* used was a plasma pool from albino rats which had been nefrectomized 24 hours earlier pH was brought to 3 6 by 2n phosphoric acid for 20 minutes at 25 degrees Celcius and subsequently to pH 7 5 by 6n NaOH After this process no measurable angiotensinase activity is found (*Bing* (1))

The *antirenin* was produced by immunisation of female albino rats of c 160 g with the above mentioned hog renin which was given intraperitoneally with Freund s complete adjuvans as stated by *Haas* (5) so that in 1 ml physiological saline the animals were given 1 Goldblatt unit per day for the first 5 days of the week The first day however a dose of 1 GU in 0 5 ml physiological saline plus 0 5 ml of Freund s complete adjuvans was given The animals were immunized for 3 weeks in all and sacrificed on the 22nd day The angiotensinase activity was reduced as described above

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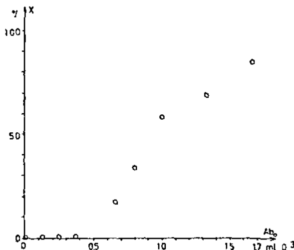


Fig 1

The concentration of the renin antirenin complex (x) is plotted against varying starting concentrations of antirenin (Ab_0) The starting concentration of renin (Ag_0) is kept constant The reaction time is 2 min at 37 degrees Celcius By comparing with Fig 2 it is seen that the inhibition is too small particularly for the lowest concentrations

Titration of the Antirenin

Renin is mixed with antirenin, and substrate is added in such a way that the non neutralized part of the renin can be determined We have chosen a small concentration of renin as compared with the concentration of substrate which has been made high by using plasma of nefrectimized rats (*Bing* (1))

100 mvl of renin 0 5 GU/ml, containing 2 per cent human albumin having no angiotensinase activity and 100 mvl of antiserum dilution of varying concentration (0 2 per cent) with one drop of tuloene added to it and a glass bead is mixed mechanically for 18 hs at 4 degrees Celcius The diluent used is trisbuffer pH 7 5 After pre incubation of the mixture for 10 minutes and admixture of 1 3 ml of trisbuffer pH 7 5 and 0 5 ml of substrate it is incubated for 10 minutes at 37 degrees Celcius Reaction is stopped by boiling 10 minutes after acidification to pH 5 with 2 n HCl Centrifugation to clear liquid and freezing of the supernatant to —20 degrees Celcius after pH has been brought to 7 5 with 2 n NaOH The concentration of angiotensin is determined biologically in a barbital and pentolinium treated rat by direct measuring of blood pressure The determination is made by comparing the pressor response with that of a standard dilution of 100 ng synthesis angiotensin Ciba/ml The single injection varies from 2 to 6 ng angiotensin corresponding to a sensitive section of the logaritmic dose response curve The concentration of the renin antirenin complex at the time = t (x) is measured as the difference between the initial concentration of renin (Ag_0) and the free concentration of renin (Ag_0 x) In order to determine (Ag_0) with the greatest possible exactitude a big pool of substrate has been produced and each analysis of (Ag_0-x) is followed by control tests of Ag_0 which varies only ± 10 per cent for each test

Titration of the complement as described by *Kabat & Mayer* (10) with electro photometric determination of the degree of haemolysis of the sensibilized sheep erythrocytes

Titration of renin antirenin by complementfixation as described by *Kabat & Mayer* (10) The degree of haemolysis however is measured electrophotometrically

Euglobulinsedimentation of the substrate 2 ml of substrate pool is dialysed (2432 Viskin) for 18 hs against 1 5 liters (three shiftings) af glass destilled water with a conductivity of 60 MHO and pH 6 5 The sediment is removed by centrifugation at 4 degrees Celcius and 2500 r p m for 1 hour

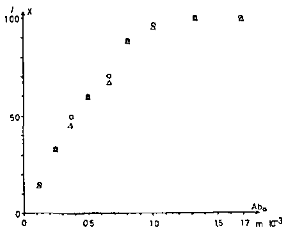


Fig 2

The concentration of the renin antirenin complex (x) is plotted against varying starting concentrations of antirenin (Ab_0) The starting concentration of renin (Ag_0) is kept constant The reaction time is 18 hs at 4 degrees Celcius The two series of values being obtained on different days

RESULTS

1 *The kinetic of the Renin Antirenin System*

Equilibrium between antigen and antibody $Ag + Ab \underset{k^2}{\overset{k^1}{=}} AgAb$ is obtained by mixing antigen (= enzym = renin) with antibody (= antirenin) The concentration of antigen at the start of the reaction (Ag_0) is kept constant and the concentration of the antibody at the start of the reaction is varied The complex concentration (x) is measured as the difference between Ag_0 and the concentration of free antigen (Ag)

If x is plotted against Ab_0 after a short reaction time (2 min at 37 degrees Celcius) we get a curve as in Fig 1 representing 1 out of 5 experiments If the experiment is repeated with the same system and a reaction time of 18 hs at 4 degrees Celcius we get a curve as in Fig 2 representing 6 experiments

In 5 cases (Ag_0-x) as the function of time has been examined at 4 degrees Celcius Ab_0 has been chosen in such a way that $x_{eq} = 1/2\ Ag_0$ (Fig 3)

If the reaction is in accordance with the mass law

$$(Ag_0 - x_{eq})(Ab_0 - x_{eq}) = \frac{1}{k}\ x_{eq}$$

x_{eq} being the equilibrium concentration of AgAb the horisontal part of the curve would denote equilibrium If at this point the reaction volume is diluted 1 1 and the velocity of the reaction is increased by

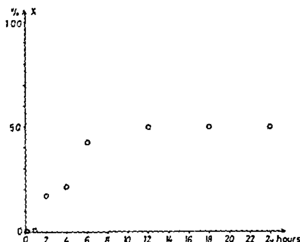


Fig 3

Inhibition percentage (x) is plotted against the time at 4 degrees Celcius The starting concentration of antirenin has been calculated so that at equilibrium the concentration of enzymatic "free" renin in equal to the concentration of renin bound in the complex

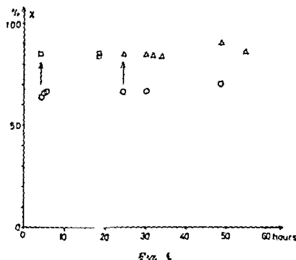


Fig 4

The inhibition is plotted against the time ○ Arrows indicate dilution 1 1 of the volume of the reaction □ indicates the reaction at 37 degrees Celcius △ indicates the reaction at 4 degrees Celcius Ab_0 is calculated so that x_{e1} is equal to 2 3 Ag_0.

raising the temperature to 37 degrees Celcius, no dissociation of the complex can be shown, not even after 18 hs It was shown by control tests that the "free" concentration of renin (Ag_0–x) of the undiluted system (Fig 3) was not changed by standing for the same time under the same circumstances (Fig 4)

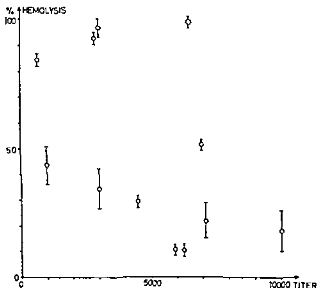


Fig 5

Each point represents an antireninserum that has been measured by units (ARIn%$_{50}$) per ml serum (the abscissa) and by its complement fixating capacity (the ordinate)

2 The Exactitude of the Antirenin Determination Method Used

In 10 experiments the concentration of antirenin is not seen to have changed after the above-mentioned angiotensinase-destroying treatment of antiserum, the titre after the treatment being as before ± 5–10 per cent Besides the use of double tests, in order to test the reproductability 8 artificial sera have been produced by mixing antiserum with normal serum, the artificial sera containing respectively 100, 90, 80, 70, 60, 50, 40, 30 per cent of antiserum For all the sera the titre has been found to be the ± 5–10 per cent calculated, corresponding to the exactitude of double tests

3 The Ability of the Renin-Antirenin System to Bind the Complement

The renin-antirenin system is found to be markedly complement fixating The complement-fixating effect was titrated for varying concentrations of renin The maximum fixation of complement is found at 0 4 GU ml With this concentration of renin the complement fixating capacity of the serum in question was determined in tests for each serum in the dilution 1 100 If this effect is compared with the enzyme neutralizing effect of the same serum, no correlation is found (Fig 5)

4. *The Possible Influence of the Complement Concentration on the Result of the Antirenin Determination*

In 4 tests the titre of the antibody was tested in a system containing a non-measurable complement content and compared with a system containing 27 $'H_{50}$/ml of guinea-pig complement The substrate has been euglobulin-sedimentated as described under "Materials and Methods" Antiserum is heated to 56 degrees Celcius for 30 minutes (*Haas* (6)) There is no measurable change of the degree of inhibition of the antibody

DISCUSSION

1 *The Kinetic of the Renin-Antirenin System*

By the above-mentioned incubation of renin in a concentration that is small as compared with the concentration of substrate we secure, above all, that the produced concentration of angiotensin per time unit is directly proportional to the "free" concentration of enzyme and that the produced concentration of angiotensin per time unit is large so that the incubation period of the reaction can be made short

By comparing Figs 1, 2, and 3 it is seen that after a reaction time of 2 minutes at 37 degrees Celcius there is no equilibrium in the system and that in percentages the weakest concentrations are those farthest from the equilibrium

Q_{10} is calculated to be 2 7 From the fixed relation between Q_{10} and *the activation energy in this range of temperature the activation energy* of the system has, according to *Arrhenius'* equation (2) been calculated to be 18 Kcal mol This is inside the range of good antibodies (*Gann* (14))

With the enzymatic measuring of the "free" concentration of renin (Ag_0 x) a dilution is produced, which does not, however, change the relation between Ag_0 and Ag_0-x *i e* the percentage inhibition of the renin, as dissociation of the complex, as shown, has not been provable under such circumstances This could be due 1) to the fact that the reaction is simply reversible, and that the renin dissociated from the complex is no longer enzymatically active, but the antigenic site yet preserved (*Haas* (5)) This is made unlikely by the fact that, as shown in Fig 4, a stationary state is brought about which furthermore is not changed even if the reaction time, 18 hs at 4 degree Celcius, is increased by another 18 hs at 37 degrees, by which process the velocity of the reaction, according to the Q_{10} of 2 7 is made c 35 times as high If this were true it would be expected that by degrees the antibody would have inactivated the enzymatic site of the "free" renin Another explanation would be 2) that the reaction is forced towards the right so that *practically all the antibody has been brought to the complex form* This is made unlikely by a curvature instead of a straight line in Fig 2 These results seem to indicate that we are dealing with a system in

which reaction kinetic applicable to simple mono- or bimolecular reactions is not applicable as a matter of course Correspondingly an attempt to determine the equilibrium constant of the reaction by the method of least squares yielded no conclusive results The reaction pattern found does however agree with that found for other antigen antibody reactions as shown by *e g* *Samuel* (12) and *Bussard* (13)

2 *The Measurement of Antirenin and the Exactitude of the Method of Analysis*

From Fig 2 it is seen that the curve runs asymptotically towards 100 per cent inhibition In accordance with this the anti renin unit (ARIn%$_{50}$) used is defined as the quantity of antibody which in the reaction system mentioned under Materials and Methods gives a 50 per cent inhibition of the renin

Thus one ARIn%$_{50}$ is equal to the quatntity of antirenin which in a total reaction volume of 200 my liters in equilibrium pH 7 5 with the concentration of salt buffer and protein medium used will give a 50 per cent enzymatic inhibition of an Ag_0 = renin concentration at the start of the reaction of 0 25 GU/ml The number of ARIn%$_{50}$ in one millilitre of serum is used as the antirenin titre of the individual sera

If it is found desirable to change the total volume of the reaction the starting concentration of renin the concentrations of the other medium and the conditions of the experiment must be kept unchanged and the number of units of ARIn%$_{50}$ measured at a 50 per cent inhibition will then be proportional to the total volume of reaction In a total volume of reaction of 1 ml *e g* a 50 per cent inhibition will require 5 ARIn%$_{50}$ If the concentrations of the reactants or the conditions of the experiments are changed it will result in another units The unit used in the present research is not directly comparable with the unit used by *Wakerlin* (9) *Haas* (5) and *Helmer* (7)

By the procedure of immunization used the titres were found to vary from 600 to 10000 ARIn%$_5$/ml serum ÷ 5 10 per cent The accuracy of the analysis corresponds to the accuracy reached in the biological test of angiotensin

3 *The Complement Fixation of the Renin Antirenin System*

Antirenin is as mentioned above resistent to heat inactivation but angiotensinogen is partly destroyed by this procedure Complement is inactivated therefore by separating C 2 and C 4 from C 1 and C 3 by dialysis against water as described above The lacking correlation between the sum of complement fixating antigen antibody systems in a serum and the renin neutralizing capacity of the same serum is explained most easily as a result of a crude renin preparation It cannot even be concluded that the renin antirenin complex fixes complement itself

The lacking influence of the complement on the neutralizing capacity of an antiserum makes the antirenin titre independent of the loss of complement by storing and freezing

SUMMARY

It has been attempted to examine the kinetic of the renin-antirenin system by applying the *indirect* analysis of renin in which the 'free' renin has been measured biologically as angiotensin

The inhibition of the enzymatically active renin as a function of the concentration of antirenin has been examined at different temperatures, and the time of equilibrium in the antigen-antibody reaction has been tested in different concentrations of antirenin The activation energy is found to be 18 Kcal/mol, Q_{10} being 2 7

On the basis of the kinetic found we evolve a method of analysis for testing low concentrations of antirenin by means of few myl antiserum

The unit used in the measurement of antirenin was $ARIn\%_{50}$, which is defined as the quantity of antirenin that in a total reaction volume of 200 myl in equilibrium, pH 7 5, the concentration of salt, buffer- and protein medium used, will give a 50 per cent enzymatic inhibition of an Ag_0 = a renin concentration at the start of the reaction of 0 25 GU ml The number of $ARIn\%_{50}$ per ml antiserum is found to vary from 600 to 10000 $ARIn\%_{50}$ per ml serum The reproductability is found to be 5–10 per cent corresponding to the accuracy reached in the method of biological analysis used The method used for inactivation of angiotensinase is without effect on the antirenin, and the concentration of angiotensinase is not measurable in the time used

This system of "crude renin and its antibody is found to be markedly complement fixing The presence of complement does not change the kinetic of the system to any measurable degree No correlation is found between the renin-neutralizing effect and its complement fixing effect

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STUDIES ON THE RENIN CONTENT OF RENAL CORTEX AUTOGRAFTS IN RATS

By

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Received 3.vi.66

The granules of the juxtaglomerular cells (JGC) are believed to be the morphological equivalents of renin (*Latta & Maunsbach* 1962, *Tobian* 1962, *Hartroft* 1963, *Endes* 1965), but according to *Bing & Kazimierczak* (1964) and *Bing* (1964) the epithelial cells in macula densa may play the primary role in the production of renin.

Investigating the morphological aspects of the renin system *Endes, Devényi & Gomba* (1963) and *Devényi, Schill & Tessman* (1961) autotransplanted pieces of rat kidney cortex subcutaneously. Whereas the tubulus showed severe regressive changes in these grafts, a considerable part of the vessels and the glomeruli remained viable and the number of juxtaglomerular granulated cells after an initial reduction became elevated. The morphological appearance of these cells suggested their functional activity. *Devényi & Dauda* investigated directly the pressure activity of extracts of these grafts and found that they after an initial depressor effect gave a pressor response (personal communication). In the present investigations we are dealing with the renin content of the transplants, estimated by more exact methods.

MATERIAL AND METHODS

30 albino rats of both sexes weighing 200 g were nephrectomised unilaterally in i.p. amytal narcosis paying particular attention to aseptic conditions. 20 minutes before the operation each animal received 50 000 I.U. Penicillin i.m. From the cortex of the removed kidney 4–5 pieces each weighing 5 to 10 mg were cut out and put into a pocket formed in the subcutis of the subscapular region. A further piece weighing 5 to 10 mg of the cortex of each kidney was stored at —20° C for control renin estimation. One of the transplants was fixed in 10 per cent formol for histological examination. The animals were kept on normal diet, receiving water ad libitum and killed by bleeding from 30 to 40 days after the transplantation.

The transplants from 12 animals were tested for renin activity by direct renin assay and the transplants from 10 other rats by the indirect method. In extracts of transplants of 2 animals the angiotensinase content was determined. The materials from 8 animals could not be used for different reasons. The cortex pieces reserved and frozen at the time of operation were assayed by direct renin determination in 9 cases.

1. For *direct renin assay* the transplants were frozen and thawed three times

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and homogenised in sod um pyrophosphat buffer (pH 5.3) containing toluene the *quantity of buffer being 49 times that of the weight of the transplants* After centrifugation the supernatants were injected i v into rats and their pressor activity was compared with the pressor effect of a standard renin solution containing 0 17 Goldblatt Unit per ml

2 For *indirect renin assay* the material prepared for direct renin determination (1) was incubated with angiotensinase free plasma of bilaterally nephrectomised rats and the quantity of angiotensin formed was tested using a standard angiotensin solution as control

3 For *angiotensinase determination* we incubated material prepared for direct renin determination (1) with a known quantity of angiotensin solution as control

For histological test we used serial sections from formol fixed paraffin embedded blocks stained by *Endes* trichrom (1963) Every 5th of the serial sections was examined The number of juxtaglomerular granulated cell groups was counted in each section and the index determined by the method of *Hartroft* (1955) The conclusions concerning the JGC content of each transplant weighing 1 5–2 mg were based on 14 18 sections of 4–5 micron thickness

In the case of 3 animals one transplant was freshly cut in a Pearse cryostat and stained for glucose 6 phosphate dehydrogenase according to *Pearse* (1960)[1]

RESULTS

Extracts of 11 of the 12 transplants tested directly showed a renin activity which was 76 per cent of the standard renin solution but the individual values varied from 24 per cent to 158 per cent

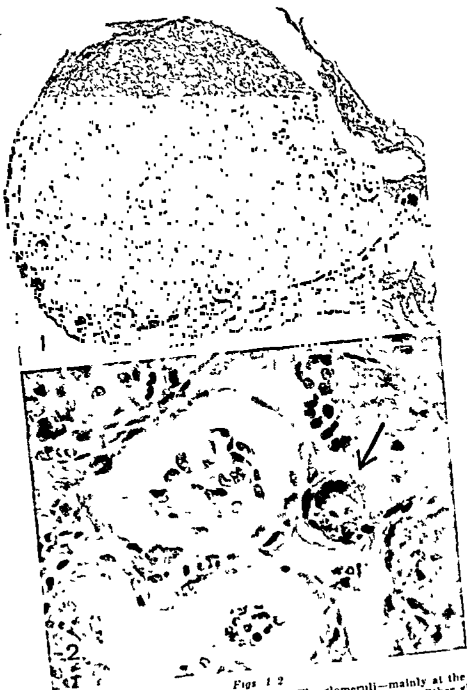
The directly measured renin activity of the extracts of cortical tissue reserved at the time of transplantation and stored in a frozen state was *on an average* 87 per cent the individual values varying from 33 per cent to 115 per cent So the average renin activity per mg of the transplants was about the same as that of the control cortex pieces No relation was found between the renin concentration of transplanted and nontransplanted cortex tissues in the same animal

Investigating with the indirect method the transplants showed renin activity The quantity of angiotensin produced by them was *on an average* 62 per cent as related to standard angiotensin solution (0 1 gamma per ml) The individual values varied from 50 per cent to 100 per cent No depressor activity was found

There was no measurable angiotensinase activity in extracts of the grafts of the two animals mentioned above

The number of the granulated juxtaglomerular cell groups per transplant (Figs 1 and 2) varied from 1 to 122 (Table 1) In three grafts we did not find JGC at all In the individual animals there was not any *quantitative relation between the JGC content of the grafts investigated* histologically and the pressure activity of extracts of the grafts tested biologically but we cannot be sure that the graft used for renin determination contained the same number of JGC as that one used for histological investigation The tubules mostly disappeared and were replaced by connective tissue infiltrated with inflammatory cells the epithelium of the surviving ones being cubic and undifferentiated It

[1] I am very grateful to Dr *J Ka im err ak* Copenhagen for his help concerning this part of my work



Figs 1 2

Fig 1 Low power of a 30 days old transplant The glomeruli—mainly at the periphery of the graft are preserved with good nuclear staining Other glomeruli are completely hyalinised The tubuli are partly obliterated, partly dilated and have a flat atrophied or a swollen, degenerated epithelium

Fig 2 High power view of a 30 days old transplant To the right of the glomerulus there is a cross section of an arteriole with heavily granulated juxtaglomerular cells (arrow) Below the glomerulus there is a dilated tubulus containing inflammatory cells and cell debris and lined by a flat, dedifferentiated epithelium

TABLE 1

Group I

Extracts Used for Direct Renin Assay

No of animal	Renin content in % of standard renin sol	Weight of tissue used for histology mg	Sum of granulated cell groups	Sum of Hartroft indices of granulated cell groups
T3	58	1.5	12	20
T4	47	2.5	0	0
T5	not evaluable	2.0	22	26
T6	79	1.6	10	20
T7	53	2.0	19	84
T8	87	1.4	0	0
T9	127	2.0	0	0
T10	158*	2.3	29	91
T14	47*	1.4	8	10
A1		2.0	34	44
A4	24	1.7	8	12
A2		1.2	10	13

Group II

Extracts Used for Indirect Renin Assay

No of animal	Angiotensin content in % of standard angiotensin sol	Weight of tissue used for histology mg	Sum of granulated cell groups	Sum of Hartroft indices of granulated cell groups
T11	100*	2.5	103	139
T15		1.5	1	1
T12	50*	2.4	88	182
T13		2.2	122	188
A3	50*	1.5	50	91
A7		2.0	61	86
A8		2.3	62	121
A5	61*	1.6	35	75
A16		1.7	1	1
A11	50*	1.9	56	107
A14		1.4	11	14

* These materials were pooled and the values represent the renin activity of the extract made from the pooled transplants

was not possible to recognise the different segments of the nephron Macula densa could never be identified Cryostat sections from three transplants did not show any glucose-6-phosphate dehydrogenase activity comparable to that of the macula densa

DISCUSSION

The kidney cortex grafts contained a significant quantity of renin The tubules showed severe regressive changes with absence of any macula

densa-like glucose-6 phosphate dehydrogenase activity, but the juxtaglomerular cells were well preserved. This is in accordance with the idea that the granules of the JGC contain renin or its precursor, and suggests that the presence of well-preserved tubular tissue is not a "sine qua non" for the production of JGC granules, i.e. the epithelioid cells seem to be able to synthetise renin. Still the possibility exists that the modified smooth muscle cells of the transplants take in renin, produced by the untouched kidney.

The JGC content of the transplants used for histological investigation was extremely variable (Table 1). This suggests that the JGC content of the transplants used for renin determination was variable too. So we could come to no quantitative conclusion concerning the relation between the quantity of JG cells and the measure of renin activity in the transplants.

SUMMARY

In rat renal cortex autotransplants, the tubules showed severe regressive changes, while the vessels and glomeruli were partly preserved and in the wall of the former juxtaglomerular apparatus granulated cells were visible after 30 days. The transplants had a considerable renin content. The relation between the renin content of the transplants and JG cells, tubular tissue and renin produced by the intact kidney of the animal is discussed.

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EXPERIMENTAL INFECTION WITH TICK BORNE VIRUS IN *CLETHRIONOMYS GLAREOLUS, APODEMUS FLAVICOLLIS, APODEMUS SYLVATICUS* AND *MUS MUSCULUS*

2 *Serological Studies*

By

GEROLF VON ZEIPEL and ZDENKA HEIGL[1]

Received 18 iv 66

The virological events following the infection with TBE virus in four species of wild rodents were reported on in a previous paper (*Heigl & von Zeipel* 1966) The present investigation is devoted to the antibody response of these rodents as followed during a long period of time after the infection Such a comparison has apparently not been carried out before in a quantitative way by means of neutralization, complement fixation and haemagglutination inhibition tests

MATERIALS AND METHODS

Animals

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about 300 to 1 subcutaneous LD_{50} units of virus for white mice whereas the species *Cl glareolus* and *A flavicollis* received 300 LD_{50} units throughout In the Hypr experiment part of the animals were kept at room temperature and part at +4° C after the inoculation of virus This was also the case in the Hallboda experiment for animals of the species *Cl glareolus* and *A flavicollis*

Blood samples from wild rodents were collected and stored as described previously (*Heigl & von Zeipel* 1966)

This work was supported in part by the Swedish Medical Research Council (Project No S 356)

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The skilful technical assistance of *Miss Siri Austrin* *Miss Sigyn Montell* and *Miss Vala Skrtić* is gratefully acknowledged

Reference Sera

A pool of 10 convalescent sera from cases of tickborne encephalitis was used to standardize the antigen of the complement fixation (CF) test. The serum pool had a titre of 1/64 against two units of antigen.

The reference serum of the neutralization (NT) and haemagglutination inhibition (HI) tests was a hyperimmune serum prepared in guinea pigs given two injections of a 10 per cent mouse brain suspension of strain Hypr at an interval of 4¾ months. A serum dilution of 1/800 neutralized 100 TCD_{50} of virus; the HI titre being 1/640 against 4 units of antigen.

Tissue Culture Methods

Stationary tube cultures of Detroit 6 cells were used throughout. The preparation and maintenance of the cultures as well as the media employed have been described in detail elsewhere (*von Zeipel & Svedmyr* 1958). However the content of human serum in the outgrowth medium was reduced to 25 instead of 40 per cent. Since 1961, as mentioned in a previous report (*Svedmyr et al* 1965) we have used a subline of our original cell line which proved to be even more suitable for neutralization tests than the original line.

Virus Material for Neutralization Tests

Virus seed material of strain Hypr (9th TC passage) was prepared in bottle cultures of Detroit 6 cells. Each bottle (bottom about 40 cm²) contained 5 ml bovine amniotic fluid (BAF) with 10 per cent unheated horse serum, 0.5 per cent Bacto tryptose and antibiotics. The inoculation dose was about 10^{3} TCD_{50} of virus. The bottle was incubated for 4 days at 35° C on a rocking table to ensure that the reduced amount of medium covered the cell sheet. The rocking table made one full cycle every 5 minutes. After harvest the fluid was clarified in an angle centrifuge at 3000 rpm for 30 minutes. Storage was at —70° C.

For titration a dilution series with 10 fold steps was made in phosphate buffered saline (PBS) containing 10 per cent horse serum. From each dilution 5 tubes were inoculated with 0.1 ml per tube.

Neutralization Tests

The plasma samples were inactivated at 56° C for 30 minutes. Serial four fold dilutions of the samples starting with dilution 1/6 were tested against 100 TCD_{50} of virus. The mixture of equal parts of serum dilution and virus suspension was incubated in a water bath at 37° C for one hour and thereafter for about 18 hours at +4° C. Each mixture was tested in two tissue-culture tubes, the inoculation volume being 0.1 ml per tube.

A titration of the virus suspension was included in all tests.

The culture tubes were incubated stationarily in an inclined position at 35° C for 9 to 10 days without change of medium.

Examination for cytopathic changes was carried out daily from the 5th day. The first cytopathic changes were visible on the 5th or 6th day proceeding to complete degeneration within 24 to 48 hours. After the 7th day a break through in a culture tube was only seldom observed.

Titres are given as the reciprocal of that dilution of serum (in the initial 0.05 ml amount) which inhibited the cytopathic effect in 50 per cent of the cultures. All titres are based on the results of the final reading and calculated according to *Kärber* (1931).

A sample negative in the dilution 1/6 was given an extrapolated titre value of 3 on the assumption that dilution 1/1.5 (four fold step) was positive. Such extrapolated values however were only given to samples from infected animals, i.e. those being virus positive and/or serologically positive in at least one of the blood samples tested.

Complement Fixation Tests

Antigens. Complement fixing antigens were prepared in the same way as virus seed material. Use was however made of Roux bottles (bottom about 240 cm²) containing 20 ml BAF with 5 per cent inactivated (56° C, 30 min.) guinea pig serum. The inoculation dose was about $10^{6.7}$ TCD_{50} of virus. The clarified fluids

were inactivated with 0.2 per cent β propiolactone as described in a previous paper (*von Zeipel & Svedmyr* 1958). Storage was at —30° C.

Plasma samples. Despite their content of heparin, which is known to be anti-complementary (*Ecker & Gross* 1929, *Ecker & Pillemer* 1941), a preliminary test on a number of plasma specimens stored at —30° C in the dilution 1/3 for at least a week showed most of them to be free from anticomplementary activity in the dilution 1/6, i.e. the lowest dilution used in the main tests. Some anticomplementary specimens were encountered however. Experiments showed that such samples could be rendered suitable for the CF reaction by the addition of a small amount of chicken or mouse embryo extract according to the following procedure. Equal volumes of plasma and 3 per cent chick embryo extract (in PBS) were mixed and allowed to stand for one hour at room temperature or at +4° C over night before being further diluted in veronal buffer[1]. In order to escape the pretesting procedure and to have a uniform treatment of the samples to be compared embryo extract was added to all specimens tested by CF in this investigation.

General CF technique. CF tests were carried out according to the micro method of *Fulton & Dumbell* (1949) as modifed by *Svedmyr et al.* (1952). Serial twofold dilutions of sera were made by the drop method in order to save material. To create better conditions for the mixing of drops, the plane lucite plates were substituted by agglutination trays containing 80 hemispherical cavities, 16 mm in diameter. These plates were obtained from Prestware Ltd., South Down Works, Kingston Road, Raynes Park, London, S.W. 20. The plates were cooled in the refrigerator before the various reagents were added. Incubation was at +4° C for 18 hours. After the addition of the haemolytic system the plates were incubated at 37° C for 60 minutes. During this period they were covered with thin plastic sheets and kept separately on the shelves of the incubator. Titres are given as the reciprocal of the highest initial serum dilution giving at least 50 per cent inhibition of haemolysis. Samples from animals with proved infection which were negative in dilution 1/6 were given a maximal titre value of 3 in accordance with the extrapolation procedure described above.

Haemagglutination Inhibition Tests

Antigen. The antigen used was kindly supplied by Dr. A. Salminen, University of Helsinki, Finland. It was prepared in a continuous line of human amnion cells grown in an inhibitor free medium (*Salminen* 1962). The culture fluid harvested 8–10 days after the infection with strain Hypr was rendered non infectious by thermal inactivation after the addition of protamin sulphate. Storage was at +4° C. The haemagglutinating capacity of the antigen was 1/256 at pH 5.8, 6.1 and 6.3 in the pH series of *Clarke & Casals* (1958). All samples were tested at pH 6.1 in this investigation.

Plasma samples. Samples were adsorbed with acid washed kaolin (Amend and Co., N.Y., U.S.A.) according to *Clarke & Casals* (1958).

The samples were then adsorbed with goose erythrocytes in an ice bath for 20 min., followed by cold centrifugation at 1500 rpm for 10 minutes.

The final dilution of a sample after the adsorptions was 1/12.

Goose erythrocytes. The goose blood was drawn directly into acid citrate dextrose solution. After washing the erythrocytes were stored as an approximately 8 per cent stock suspension in dextrose gelatine veronal solution. A constant concentration of erythrocytes in the working suspensions was obtained by standardization to the same optical density at 490 mμ in a Bausch and Lomb Spectronic 20 photoelectric colorimeter. The stock suspension was in general diluted 1:40 to obtain the working suspension. This concentration, recommended by Clarke and Casals for tubes, was chosen since the cups of the plates were tube like.

General technique. Serial twofold dilutions of samples in bovalbumine borate saline, starting with dilution 1/12, were made directly in the cups of plastic plates containing 96 cups, 6 mm in diameter, with conical bottom (Cooke En-

[1] The procedure was also suitable for treatment of fresh plasma specimens which were always found to be anticomplementary. After coagulation these samples were frozen to —30° C so that the serum became separated from the coagulum on thawing. However, coagulation was not noticed in specimens stored in the frozen state before treatment with embryo extract.

gineering Co, Alexandria Virginia U S A) The antigen serum mixtures (0 05 ml of each) were incubated for 18 hours at +4° C, after which 0 1 ml of erythrocyte suspension was added to each cup The plates were kept for one hour at room temperature (+22° C) before reading

Specimens from animals with proved infection which were negative in dilution 1/12 were given an extrapolated titre value of 6

Arrangement of Blood Samples in the Tests

Sufficient amounts of preinfectious blood samples were in general available for the NT test only Furthermore, a number of specimens drawn during the first week after inoculation of virus were used up in the isolation experiments described in the preceding paper (*Heigl & Zeipel* 1966) With these exceptions, the vast majority of sera could be tested in all three reactions (NT CF and HI)

All the sera from one and the same animal were run in the same NT, CF or HI test so as to obtain a proper comparison between the samples

Representatives of different animal species were tested in each NT, CF or HI reaction to minimize the influence of methodological errors when comparing the results obtained in different species

Graphs

The trend lines of antibody titres on the graphs are drawn through points representing the geometric mean values These include the extrapolated titres given to negative specimens Specimens from animals not developing viraemia or NT antibodies are not recorded on the graphs

Statistical Method

Choice of method A certain number of the populations of titres to be compared were apparently not normally distributed In some cases, furthermore significant differences between variances were found The assumptions required for the application of the Student's t test were therefore violated for part of the material Although the t test has been shown to be relatively resistent to such violations (for references see *Boneau* 1960 *McNemar* 1962) it was considered safer to use the nonparametric Rank sum test the power of which is only little less than that of a t test when treating normal populations and which may be even more powerful for nonnormal populations (see *Dixon & Massey* 1957) [1]

The Rank sum test was applied without adjustment for ties (see *Brownlee* 1960) as the gain in power through this correction was of negligeable order for the populations of the present material

Scheme of statistical treatment In the Hypr experiment only two species (*A sylvaticus* and *M musculus*) had to be compared The Hallboda experiment comprised however four species In this experiment therefore one species (*A flavicollis*) was taken as the middle group to be compared downwards with the low titering species (*A sylvaticus*) and upwards with the high titering species (*Cl glareolus* and *M musculus*)

The comparison between the species as regards antibody production was performed in two ways

1 Comparison between species as to means of the highest antibody titres recorded independently of time for individual animals

2 Comparison between species as to means of antibody titres obtained for each of three different periods of the experiments These periods represented together a time of about 4 weeks starting approximately from the third week after inoculation of virus The periods were

period 1 Hypr test, pooled data of samples from days 15 17 and 21
Hallboda test pooled data of samples from days 14 19 and 23
period 2 Hypr test, pooled data of samples from days 25 29 and 35
Hallboda test pooled data of samples from days 27, 31 and 35
period 3 pooled data of samples from the 42nd day, representing all animals of a species remaining at the end of an experiment

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1 It may be mentioned that all data were recalculated according to the Student's t test Only exceptionally a minor change in the level of significance was observed

Only surviving animals of a species are represented during the three periods of time given above. Although not necessary for transversal comparisons between the species this arrangement makes possible longitudinal studies within the same species as regards the rising and falling tendency of the curves. It has however led to a discrepancy between the number of animals in the Tables and the Figures for animals dying before the end of an experiment are absent from the former but recorded in the latter.

The means of titres are in the Tables given as geometrical mean values.

The following symbols are used

D Difference between two groups (quotient)
GM geometrical mean
N number of samples
z standardized score of the Rank sum test

* probably significant ($P < 0.05$)
** significant ($P < 0.01$)
*** highly significant ($P < 0.001$)

RESULTS

NT antibodies against TBE virus could not be demonstrated in any of the preinfectious blood samples of the rodents under the conditions of the test. The CF and HI reactions on these specimens were also negative to the extent carried out.

A close correspondence as to appearance, rise and decline was found in the NT as well as in the CF and HI reactions between antibody titres obtained in animals kept at room temperature and at + 4° C. Means and variances were closely similar. Therefore the results recorded in equivalent groups of animals at the two temperatures could be pooled and a reduced number of larger groups obtained.

The data recorded in the titration experiments were also pooled since similarly no differences as regards antibody formation were found in animals infected by high or low doses of virus (i. e. 330 to 1 LD_{50} for white mice).

Neutralization Tests

The titres of NT antibodies in *A. sylvaticus* and *M. musculus* after inoculation of Hypr virus are recorded in Figs. 1 and 2, the antibody response in the same species as well as in *A. flavicollis* and *Cl. glareolus* after infection with Hallboda virus is shown in Figs. 3 to 6.

The earliest samples tested originated, as seen in the Figures, from day 7 (Hallboda experiment) or day 9 (Hypr experiment). Already at this stage at least some representatives of all species were positive. During days 9–13 in the Hypr test the same proportion (about 50 per cent) of positive specimens was found in both *A. sylvaticus* and *M. musculus*. During a similar period (days 7–12) of the Hallboda experiment, on the other hand, different figures of positivity were obtained for the various species: *A. sylvaticus* 43 per cent, *A. flavicollis* 66 per cent, *Cl. glareolus* and *M. musculus* about 100 per cent. After two weeks all *A. flavicollis*, *Cl. glareolus* and *M. musculus* became positive, whereas

gineering Co Alexandria Virginia USA) The antigen serum mixtures (0 05 ml of each) were incubated for 18 hours at +4° C, after which 0 1 ml of erythrocyte suspension was added to each cup The plates were kept for one hour at room temperature (+22° C) before reading

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All the sera from one and the same animal were run in the same NT, CF or HI test so as to obtain a proper comparison between the samples

Representatives of different animal species were tested in each NT CF or HI reaction to minimize the influence of methodological errors when comparing the results obtained in different species

Graphs

The trend lines of antibody titres on the graphs are drawn through points representing the geometric mean values These include the extrapolated titres given to negative specimens Specimens from animals not developing viraemia or NT antibodies are not recorded on the graphs

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period 3 pooled data of samples from the 42nd day representing all animals of a species remaining at the end of an experiment

1 It may be mentioned that all data were recalculated according to the Student's t test Only exceptionally a minor change in the level of significance was observed

a few *A sylvaticus* remained negative, one for at least 23 days (Fig 3, Hallboda test), another for at least 35 days (Fig 1, Hypr test) All specimens collected after 42 days contained NT antibodies, however

The different levels of NT antibodies during the last four weeks of the experiments, as illustrated in the Figures, give an impression of substantial differences between several species The validity of this observation was subjected to statistical analysis in the following way

TABLE 1

NT Antibodies in Rodents after Infection with Hypr Virus Comparison between Species as to Means of Titres Obtained at Various Intervals of Time after Inoculation

Species	Time after inoculation 15 17, 21 days (period 1)			25 29 35 days (period 2)			42 days (period 3)		
	N		GM	N		GM	N		GM
A sylvaticus	14		15	14		31	15		46
M musculus	17		54	17		340	17		550
Comparison	D	z	P	D	z	P	D	z	P
A sylv M musc	3.5	2.22	*	11	3.89	***	12	4.19	***

TABLE 2

NT Antibodies in Rodents after Infection with Hallboda Virus Comparison between Species as to Means of Titres Obtained at Various Intervals of Time after Inoculation

Species	Time after inoculation 14 19 23 days (period 1)			27 31 35 days (period 2)			42 days (period 3)		
	N		GM	N		GM	N		GM
A sylvaticus	15		16	13		43	15		63
A flavicollis	27		62	27		140	27		240
Cl glareolus	19		430	19		550	19		550
M musculus	29		100	28		440	28		530
Comparisons	D	z	P	D	z	P	D	z	P
A flav A sylv	*3.9*	*3.07*	**	*3.3*	*2.58*	**	*3.7*	*3.58*	***
A flav -Cl glar	6.9	4.39	***	3.9	4.22	***	2.3	3.52	***
A flav M musc	1.6	1.47		3.1	4.19	***	2.2	3.75	***

The analysis of data of the Hypr experiment pooled according to periods of time (Table 1) showed that the mean titres recorded in *A sylvaticus* were considerably lower than in *M musculus* Significant differences between these two species were also found in the Hallboda

test. The analysis of the latter experiment (Table 2) furthermore demonstrated that *A. sylvaticus* had the lowest antibody titres of all four species. The closely related species *A. flavicollis* apparently occupies an intermediate position in this respect, differing significantly from *A. sylvaticus* as well as from the high titering species of *Cl. glareolus* and *M. musculus*. About the same means of titres were recorded in the two last mentioned species except for the early parts of the curves (Table 2, period 1) where a steeper rise in antibody production to the maximum plateau was noticed in *Cl. glareolus*. Thus the mean titre found in this species during "period 1" was 4 times higher than that obtained in *M. musculus* ($z = 2.78$ *)

TABLE 3

NT Antibodies in Rodents after Inoculation with Hypr Virus. Comparison between Species as to Means of Highest Titres of Individual Animals Recorded Independently of Time

Species	N		GM	Antibody titre 6	12	24	48	96	192	384	5 6	768
A. sylvaticus	15		55	1†	2	2	4	3	1	1		1
M. musculus	17		600						2	1	3	11
Comparison	D	z	P									
A. sylv.–M. musc.	11	4.25	***									

† Figures mark the number of animals with a certain titre

TABLE 4

NT Antibodies in Rodents after Infection with Hallboda virus. Comparison between Species as to Means of Highest Titres of Individual Animals Recorded Independently of Time

Species	N		GM	Antibody titre 12	24	48	96	192	384	768	1536
A. sylvaticus	16		71	2†	3	2	3	5	1		
A. flavicollis	27		240			1	2	16	3	5	
Cl. glareolus	19		720					2	1	13	3
M. musculus	28		620					3	4	20	1
Comparisons	D	z	P								
A. flav.–A. syl.	3.4	3.35									
A. flav.–Cl. glar.	3.0	4.14									
A. flav.–M. musc.	2.6	4.28									

† Figures mark the number of animals with a certain titre

The comparison between the means of the highest titres obtained in individual animals independently of time (Tables 3 and 4) gave the same differentiation between the species as demonstrated above for data pooled according to periods of time. Thus *A. flavicollis* had a mean

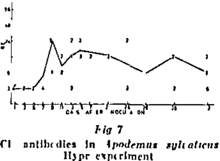


Fig 7
CF antibodies in *Apodemus sylvaticus* Hypr experiment

Fig 8
CF antibodies in *Mus musculus* Hypr experiment

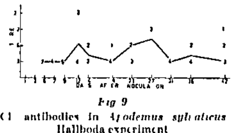


Fig 9
CF antibodies in *Apodemus sylvaticus* Hallboda experiment

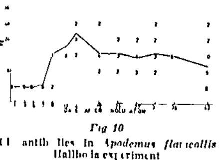


Fig 10
CF antibodies in *Apodemus flavicollis* Hallboda experiment

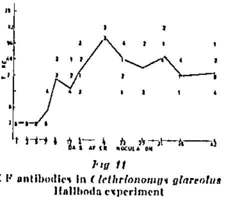


Fig 11
CF antibodies in *Clethrionomys glareolus* Hallboda experiment

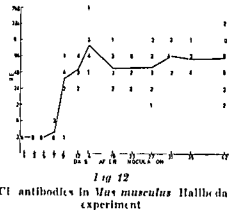


Fig 12
CF antibodies in *Mus musculus* Hallboda experiment

* Figures mark the number of animals with a certain titre

titre 3 4 times higher than had *A sylvaticus*, whereas *Cl glareolus* and *M musculus* had 3 and 2 6 times higher titres respectively than *A flavicollis* These differences were statistically significant

Tables 3 and 4 also give information about the highest titre reached in individual animals This indicates at the same time the rise in titre since all animals as mentioned above were negative in specimens drawn before the inoculation of virus

It should be pointed out that, with exception for one *A sylvaticus* and one *Cl glareolus*, not a single 42 day specimen showed a ≥ four-fold drop in NT titre, actually it is possible that the maximal titre level was not always reached at this time On the other hand a statistically insignificant reduction in the mean titres to about 2/3 of the 42 day values was found in specimens drawn 110 days after inoculation of Hallboda virus into 5 *A sylvaticus* and 6 *M musculus* The five specimens of *A sylvaticus* all happened to have high titres

The NT antibody response of *A sylvaticus* on the one hand and *M musculus* on the other was apparently similar whether the animals had been inoculated with Hypr or Hallboda viruses

Complement Fixation Tests

The titres of CF antibodies in *A sylvaticus* and *M musculus* after inoculation of Hypr virus are recorded in Figs 7 and 8, the antibody response in all four species after infection with Hallboda virus is shown in Figs 9 to 12

As illustrated by the curves and the analyses in Tables 5 and 6 the animal species could be arranged on the basis of the CF results in the same order regards weak and strong antibody production as was arrived at for NT antibodies The mean titres pooled according to periods of time showed significant differences between *A sylvaticus* and *M musculus* in the Hypr experiment Also the differences in the Hallboda experiment were significant between *A flavicollis* and *A sylvaticus* as well as between *A flavicollis* and *Cl glareolus*-*M musculus*

TABLE 5

CF Antibodies in Rodents after Infection with Hypr virus Comparison between Species as to Means of Titres Obtained at Various Intervals of Time after Inoculation

Species	Time after inoculation 15 17 21 days (period 1) N		GM	25 29 35 days (period 2) N		GM	42 days (period 3) N		GM
A sylvaticus	15		12	14		9 4	14		6 6
M musculus	17		41	17		61	17		64
Comparison	D	z	P	D	z	P	D	z	P
A sylv M musc	3 4	3 40	***	6 5	3 89	***	9 6	4 23	***

TABLE 6

CF Antibodies in Rodents after Infection with Hallboda Virus Comparison between Species as to Means of Titres Obtained at Various Intervals of Time after Inoculation

Species	Time after inoculation					
	14 19 23 days (period 1)		27, 31, 35 days (period 2)		42 days (period 3)	
	N	GM	N	GM	N	GM
A sylvaticus	15	4 3	15	5 0	16	3 6
A flavicollis	27	18	27	12	27	8 4
Cl glareolus	19	64	19	39	19	28
M musculus	28	85	28	79	28	91

Comparisons	D	z	P	D	z	P	D	z	P
A flav.-A sylv	4.2	4.34	***	2 5	3 48	***	2 3	3 14	**
A flav Cl glar	3 6	3 59	***	3 1	3 65	***	3 3	3 76	***
A flav M musc	4 7	4 98	***	6 4	5 83	***	11	5 86	***

The intermediate position of *A flavicollis* was again confirmed on comparison of the means of the highest individual titres obtained independently of time in each species (Table 8) Thus the mean titre of *A flavicollis* was found to be about 4 times higher than that of *A sylvaticus*, and yet 3 and 5 times lower than the means of *Cl glareolus* and *M musculus* respectively These differences were statistically significant

The presence of CF antibodies could not be demonstrated in specimens drawn before the 7th day

A sylvaticus was the only species in which animals were encountered that did not develop CF antibodies under the conditions of the test The number of such animals was 1 out of 15 in the Hypr experiment and 8 out of 16 in the Hallboda experiment (Tables 7 and 8) It should be mentioned that certain other *A sylvaticus* were positive in only one or two of the samples tested This was also true for one *A flavicollis*

TABLE 7

CF Antibodies in Rodents after Infection with Hypr Virus Comparison between Species as to Means of Highest Titres of Individual Animals Recorded Independently of Time

Species	N	GM	Antibody titre							
			3	6	12	24	48	96	192	384
A sylvaticus	15	17	1†	1	6	5		2	–	
M musculus	17	79			1	2	3	7	3	1

Comparison	D	z	P
A sylv M musc	4 5	3 61	***

† Figures mark the number of animals with a certain titre

TABLE 8

CF Antibodies in Rodents after Infection with Hallboda Virus Comparison between Species as to Means of Highest Titres of Individual Animals Recorded Independently of Time

Species	N	GM	Antibody titre 3	6	12	24	48	96	192	384	768
A sylvaticus	16	6 3	8†	1	5	2				–	–
A flavicollis	27	27			9	8	8	1	1		–
Cl glareolus	19	80	–		2	1	4	7	3	2	
M musculus	28	140		–			3	12	10	2	1

Comparisons	D	z	P
A flav A sylv	4 3	4 21	***
A flav Cl glar	3 0	3 60	***
A flav-M musc	5 1	5 67	***

† *Figures mark the number of animals with a certain titre*

Peak titres were reached at somewhat different times in the various species Such a difference was also noted between the subgroups tested with Hypr or Hallboda viruses (*A sylvaticus, M musculus*) The antibody peak was noticed early in Hypr-infected *A sylvaticus* (9th day, Fig 7) but relatively late in the systems of *Cl glareolus*—Hallboda virus (19th day, Fig 11) and *M musculus*—Hypr virus (17th–25th day, Fig 8) With exception for *Cl glareolus* the CF peak occurred about two weeks earlier than the peak of NT antibodies

Soon after the peak was reached the CF curves apparently had a falling tendency in all species except in *M musculus* Thus a four-fold or greater decrease in titre was recorded at 42 days in about 50 per cent of the animals belonging to *A sylvaticus* (Hallboda and Hypr groups taken together), *A flavicollis* and *Cl glareolus*, the corresponding figure being only 14 per cent in *M musculus* A decrease in titres in the species mentioned was further substantiated on comparison between the titres of the 42nd day (Tables 5 and 6, period 3) and the highest individual titres recorded independently of time (Tables 7 and 8) The differences between these two means given together with the level of significance are as follows *A sylvaticus*—Hypr virus (2 6 times, z = 2 57*), *A flavicollis* (3 3 times, z = 4 35***), *Cl glareolus* (2 9 times z = 2 92**) No difference was obtained within the group of *A sylvaticus* inoculated with Hallboda virus This could, however, hardly be expected as both series contained few quantitative data due to the presence of a considerable number of negative animals

A comparison in the above species between the values obtained for "period 1" and those for day 42 (Tables 5 and 6) indicated a decrease in titres of some significance only in *A flavicollis* (2 2 times z = 2 93**) and in *Cl glareolus* (2 3 times z = 2 30*) Evidently such a longitudinal comparison was less apt to reveal a falling tendency of the

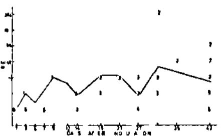


Fig 13
HI antibodies in Apodemus sylvaticus
Hypr experiment



Fig 14
HI antibodies in Mus musculus Hypr
experiment

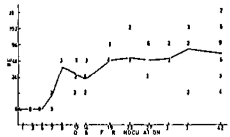


Fig 15
HI antibodies in Apodemus sylvaticus
Hallbola experiment

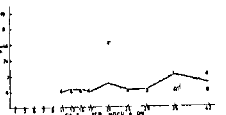


Fig 16
HI antibodies in Apodemus flavicollis
Hallbola experiment



Fig 17
HI antibodies in Clethrionomys glareolus
Hallbola experiment

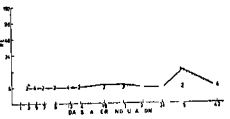


Fig 18
HI antibodies in Mus musculus Hallbola
experiment

* Figures mark the number of animals with a certain titre

Dotted lines — antibody curves of individual animals

titres since not all animals reached their highest individual value during "period 1"

As a result of the decrease in CF antibodies negative 42 day specimens were found in all species, except in *M musculus*, the proportions being as follows *A sylvaticus* (Hypr and Hallboda groups) 63 per cent (animals negative in all specimens included), *A flavicollis* 30 per cent, *Cl glareolus* 5 per cent

The discrepancy between the stability of CF antibody in *M musculus* and the decreasing titres of the other species is further illustrated by the fact that the mean CF titre for 6 *M musculus* bled 110 days after inoculation of virus was only 40 per cent lower than the mean value recorded on the 42nd day

Haemagglutination-Inhibition Tests

The results of the HI tests in *A sylvaticus* and *M musculus* after inoculation of Hypr virus are recorded in Figs 13 and 14, the outcome of the tests in all species after infection with Hallboda virus is shown in Figs 15 to 18

Obviously the HI reaction gave a similar differentiation between the species as regards low and high antibody production (Tables 9, 10, and 11) as did the NT and CF tests There was, however, one exception Thus *Cl glareolus* apparently occupied an intermediate position between *A flavicollis* and *M musculus* not differing significantly from the former species (except for "period 1", Table 9) nor from the latter species (except for day 42, Table 9, D = 2 8 times, z = 3 06**)

Contrary to the findings in the CF tests positive HI results were encountered as early as on the 3rd and 5th day in *A flavicollis* and *Cl glareolus* From some of these animals specimens drawn prior to inoculation were available These proved to be negative in 2 *A flavicollis* having titres of 48 and 96 on the 3rd day This was also true

TABLE 9

HI Antibodies in Rodents after Infection with Hallboda Virus Comparison between Species as to Means of Titres Obtained at Various Intervals of Time after Inoculation

Species	Time after inoculation: 14 19 23 days (period 1)			27 31 35 days (period 2)			42 days (period 3)		
	N		GM	N		GM	N		GM
A flavicollis	26		19	26		22	26		21
Cl glareolus	19		45	18		43	19		24
M musculus	28		38	27		59	28		68
Comparisons	D	z	P	D	z	P	D	z	P
A flav-Cl glar	2 3	2 55	*	1 9	1 13				
A flav M musc	1 9	2 73	**	2 7	3 15	**	3 2	3 67	***

for 2 further *A flavicollis* positive (titres 12 and 24) on the 5th day as well as for one *Cl glareolus* (titre 12) from the same day Positive results in *M musculus* were not recorded until the 7th day in the Hallboda experiment, in the Hypr experiment specimens drawn before the 9th day were not tested The first positive *A sylvaticus* was met with on the 13th day

TABLE 10

HI Antibodies in Rodents after Infection with Hypr Virus Comparison between Species as to Means of Highest Titres of Individual Animals Recorded Independently of Time

Species	N	GM	Antibody titre 6	12	24	48	96	192	384
A sylvaticus	15	10	7†	7	--		--	1	--
M musculus	17	75			4	4	5	2	2

† Figures mark the number of animals with a certain titre

TABLE 11

HI Antibodies in Rodents after Infection with Hallboda Virus Comparison between Species as to Means of Highest Titres of Individual Animals Recorded Independently of Time

Species	N		GM	Antibody titre 6	12	24	48	96	192	384
A sylvaticus	15		7 5	12†	2		1	--		
A flavicollis	27		32	3	4	8	8	1	1	2
Cl glareolus	19		48	1	3	4	5	1	3	2
M musculus	28		79	--	3	2	6	9	5	3
Comparisons	D	z	P							
A flav–A sylv	4 2	4 08	***							
A flav M musc	2 5	3 11	**							

† Figures mark the number of animals with a certain titre

A number of animals did not develop HI antibodies under the conditions of the test Thus out of 30 *A sylvaticus* not less than 19 animals were negative in all specimens and further 9 were positive, in one or two samples only, in the lowest dilution tested (*i e* 12) Altogether titres of some significance were obtained in 2 animals The individual curves for the positive animals are shown in Figs 13 and 15 The proportion of negative animals in *A flavicollis* and *Cl glareolus* was 3 out of 27 and 1 out of 19 respectively Two further animals one of each species, showed a positive reaction of the lowest order (*i e* 12) in only one of the samples tested Contrary to the other species all *M musculus* developed HI antibodies although these appeared late (after more than 3 weeks) in two animals of the Hypr experiment

A four-fold or greater decrease in titre was recorded on the 42nd day in all species in the following proportions, *A. sylvaticus* 1/2, *A flavicollis* 5/26, *Cl glareolus* 6/19, *M musculus* 1/28 A comparison between the mean value obtained at 42 days (Table 9) and the mean of the highest titres recorded independently of time (Table 11) did, however, not show a significant drop in titre in any of the species, *A sylvaticus* for obvious reasons not being tested

As in the CF test negative 42 day specimens were found in the HI test in all species, except in *M musculus*, the proportion being as follows: *A sylvaticus* 79 per cent, *A flavicollis* 19 per cent, *Cl glareolus* 21 per cent

The stability of the HI antibodies was followed in a small number of animals Specimens drawn after 42 days and 110 days showed no change in titre in 1 *A. sylvaticus*, 1 *A flavicollis* and 6 *M musculus* Specimens from the latter group drawn after 175 days gave, however, a mean value 40 per cent lower than that of the 42nd day

NT, CF and HI Antibodies in Individual Animals

As shown above the NT, CF and HI tests gave a similar differentiation between the species as to low, intermediate and high antibody response This does not mean, however, that the three tests ran parallel in individual animals, giving either a low, an intermediate or a high titre In fact the individual variations were large and there was no correlation between the peak values of the three antibody types reached in the same individual

Influence of Age and Sex on the Antibody Response

As shown in a previous paper (*Heigl & von Zeipel* 1966) male animals predominated the group of *A sylvaticus* whereas animals of the other species were almost equally distributed to sex

The age composition of all species was furthermore heterogenous

No correlation was, however, found between the magnitude of the serological response and age or sex, respectively

DISCUSSION

The antibody formation of all animals studied must be interpreted as a response to a primary infection with TBE virus for no antibodies were detected in any of the blood specimens drawn prior to the inoculation of virus The virus dose, ranging from one to about 300 subcutaneous LD_{50} units for white mice, was furthermore most probably too low to stimulate per se the antibody production

The similarity between the antibody curves obtained for animals kept at room temperature and at + 4° C may need some further comment It is well known that normothermic animals exposed to cold

react with an elevated metabolic rate This is moreover accompanied by an increase in protein turnover which in rabbits has been shown to result in an accelerated rate of antibody decay (*Trapani & Campbell* 1959, *Trapani* 1960) No evidence of the latter phenomenon was under the conditions of the tests found in the present investigation in any of the species tested As suggested by Trapani this does, however, not exclude the presence of an increase in the antibody decay of the cold exposed animals as such a decay may have been obscured by an increased antibody production resulting in a net immune response similar to that obtained in animals kept at room temperature If the latter explanation might be valid for the outcome of the present investigation can however not be decided upon

It is an interesting finding that the three species of *Muridae* showed such great differences in the quantitative formation of antibodies so as to be classified as high titering (*M musculus*), intermediate titering (*A flavicollis*) and low titering (*A sylvaticus*) The difference between *A sylvaticus* and *A flavicollis* is especially remarkable considering their very close relationship *Cl glareolus*, the only species of *Microtidae* tested showed an antibody response of the same order as *M musculus* in the case of neutralizing (NT) antibodies but gave slightly lower titres than this species in the complement fixation (CF) and haemagglutination inhibition (HI) tests It may in this connection be mentioned that the low titering species (*A sylvaticus* and *A flavicollis*) in a preceeding part of this investigation (*Heigl & von Zeipel* 1966) were found to have a somewhat lower and shorter lasting viraemia than the two high titering species Yet no difference in sensitivity to minimal doses of virus was noted No further data are, however, available supporting a hypothesis of a correlation between the magnitude of viraemia and the magnitude of antibody response

The observed differences between the species as to the magnitude of the antibody response must be regarded as reasonably proved, being established by three serological methods (NT, CF and HI) and found to be present at various intervals after the inoculation of virus The differences may in fact be even greater than indicated in the Tables as the procedure of giving a negative specimen a highest possible value probably has brought about too high a mean value for the low titering species, in the first place to *A sylvaticus* and secondly to *A flavicollis* The only dissimilarities known to be present in the composition of the various species refer to age and sex These factors were, however, not found to have influenced the results Yet the populations tested originate from relatively narrow areas and the differences found can hardly be regarded as characteristic features of the various species unless confirmed on populations from other localities of this and preferably other countries

A further difference between the species that may be briefly touched upon is the significant decrease in titre of the CF antibodies noticed in

A. sylvaticus, *A. flavicollis* and *Cl. glareolus* towards the end of the 6 week period after the inoculation of virus. At that time the CF antibodies in *M. musculus* still remained at a maximum level. If this difference as well as the general differences in antibody response have some relation to possible variations in production and life time of the antibodies in different species and/or variations of a possible shift in the composition of the immune globulins as to the fractions 19 S and 7 S must await a further investigation.

The early phase of the antibody production could not be subjected to an adequate statistical analysis to compare the appearance of antibodies in the different species as the number of observations during this short period were too few to permit the detection of minor time differences. The presence of NT antibodies was moreover not tested until the 7th or the 9th day. It may however be noted that some representatives of the low titering species were found to develop antibodies as early as animals of the high titering species in the three serological tests with exception for *A. sylvaticus* in the HI test. On the other hand evidently a number of animals belonging to the low titering species either became positive comparatively late (some *A. sylvaticus* even in the NT tests) or remained negative throughout the experiments under the conditions of the CF and HI tests.

Whatever the explanation for the failure of the CF and HI tests may be the fact remains that they must be regarded as far less reliable than the NT test in revealing passed infections of TBE virus in some of the animal species investigated. This view is also supported by the relatively rapid fall of the CF antibodies shown to occur in several species. It is therefore obvious that the results of the present study have a direct bearing to the planning as well as the analysis of serological field investigations comprising the animal species studied.

SUMMARY

The antibody response in four species of wild rodents was followed over a period of six weeks after the subcutaneous inoculation of TBE virus, usually in a dose of 75 or 300 subcutaneous LD_{50} for white mice. The response was similar whether the animals were kept at room temperature or at + 4° C.

A final number of 19 *Cl. glareolus*, 27 *A. flavicollis*, 31 *A. sylvaticus* and 45 *M. musculus* were examined over the whole period.

The quantitative evaluation by neutralization (NT), complement fixation (CF) and haemagglutination inhibition (HI) tests of blood samples drawn serially from each of the animals revealed significant differences in antibody titre between the species. Generally the lowest values were found in *A. sylvaticus*, the highest in *Cl. glareolus* and *M. musculus*. The species *A. flavicollis* occupied an intermediate position. Both *A. sylvaticus* and *M. musculus* could be infected with about one

s c LD_{50} for white mice the subsequent antibody response being similar to that of animals receiving 300 LD_{50}

Neutralizing antibodies were demonstrated in all animals of the four species CF antibodies in all but 30 per cent of *A sylvaticus* A varying proportion of animals belonging to the three species other than *M musculus* did not develop HI antibodies under the conditions of the tests This together with a subsequent drop of the CF titres occurring mainly within the species *A sylvaticus*, *A flavicollis* and *Cl glareolus*, resulted in a high proportion of such animals negative in these tests 6 weeks after inoculation of virus By that time the approximate frequencies of animals negative in CF and HI tests were as follows *A sylvaticus*, CF 63 per cent HI 79 per cent *A flavicollis* CF 30 per cent HI 19 per cent *Cl glareolus* CF 5 per cent HI 32 per cent There was no significant drop of NT titres over the time intervals studied *i e* up to 110 days

The NT reaction being a reliable test for passed infection with TBE virus in these rodents, appears to be the method of choice for most ecological work

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ACTIVATION OF C'1 TO C'1 ESTERASE ON GEL FILTRATION ON SEPHADEX G-200

Studies on Normal Human Serum, Euglobulin and Hereditary Angioneurotic Edema Serum

By

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Received 27 iv 66

It is generally accepted that the C'1 component of complement occurs in serum in a hemolytically inactive form On interaction with antigen antibody complexes C'1 is converted to an active enzyme which has been called C'1a, activated C'1 or C'1 esterase (*Becker* 1956, 1961, *Lepow et al* 1956a, b, *Lepow* 1959, *Rapp & Borsos* 1963a) *Borsos et al* (1964) have demonstrated that C'1 attaches itself to the surface of the antigen antibody complex in an inactive form where it is then activated

By chromatography on DEAE cellulose in the presence of sodium EDTA C'1 has been separated into three subunits, C'1q, C'1r and C'1s (*Lepow et al* 1963) C'1q has been identified as the S11 component (*Muller-Eberhard* 1961, *Muller-Eberhard & Kunkel* 1961) and C'1s as the C'1 proesterase (*Lepow et al* 1963) C'1r has not been identified as any previously known component and its nature is still obscure

C'1 esterase inactivates the C'4 and C'2 components in the fluid phase (*Lepow et al* 1956a, b, *Muller-Eberhard & Lepow* 1965) and hydrolyzes some synthetic esters *e g* N-acetyl-L-tyrosine-ethylester (ATEe) (*Lepow et al* 1956a, b, *Ratnoff & Lepow* 1957)

There is some confusion concerning the term C'1 esterase it may mean the activated macromolecular C'1, C'1a, or activated C'1s Besides inactivating C'4 and hydrolyzing ATEe macromolecular C'1a possesses hemolytic C'1 activity, the activated C'1s, a split product of the macro molecular C'1a, destroys C'4 and hydrolyzes ATEe but has no hemolytic activity unless combined with the other subunits (C'1q and C'1r) of the C'1 macromolecular complex In this paper the activated C'1 macromolecule will be termed C'1a the activated low molecular split product, activated C'1s

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An inhibitor of C'1a and activated C'1s, the C'1 esterase inhibitor, occurs in normal serum (*Ratnoff & Lepow* 1957, *Levy & Lepow* 1959). Sera from patients with hereditary angioneurotic edema (HANE) lack C'1 esterase inhibitor and are able to hydrolyze ATEe (*Donaldson & Evans* 1963, *Donaldson & Rosen* 1964).

The present paper reports the results obtained on fractionation of normal human serum, of euglobulin preparations from normal human serum and of serum from a patient with HANE performed under various conditions on Sephadex G 200. The fractions were studied for C'1, C'1a and for their ability to inactivate C'4 and to hydrolyze ATEe. When gel filtration was performed in the presence of calcium ions, the localization of these activities differed from that obtained when the gel filtration was performed in the presence of sodium EDTA.

MATERIAL AND METHODS

Normal sera from blood donors were pooled and kept at —60° C until used.

Euglobulin from normal human serum was prepared according to *Haines & Lepow* (1964). The euglobulin preparations were incubated at 37° C for 15 minutes and then dialysed against the salt solution used for gel filtration. The preparations were either used immediately for gel filtration or stored at —60° C until used.

C'1 esterase activated C'1s partially purified by chromatography on DEAE cellulose according to *Haines & Lepow* (1964). Only the first purification cycle was performed.

Serum from a patient with hereditary angioneurotic edema (HANE) was obtained in symptom free period. The serum was stored at —60° C. No C'1 esterase inhibitor but active C'1 esterase (ATEe hydrolyzing activity) was found in this serum.

Triethanolamine buffer 0.015 M pH 7.4 containing 0.135 M sodium chloride.

Veronal buffer (VB) was prepared according to *Kabat & Mayer* (1961).

Isotonic Sucrose Veronal buffer ionic strength 0.065 was prepared according to *Rapp & Borsos* (1963 b).

Salts of ethylene diamine tetracetic acid (EDTA). Reagent grade Na_2H_2EDTA 1×10^{-3} M in 0.15 M sodium chloride was used as eluent in some of the gel filtration experiments.

MgNa-EDTA was added to normal human serum for use as a source of C'2, C'3 and C'4 (see below).

N acetyl L tyrosine ethyl ester (ATEe) from Calbiochem, Luzerne, Switzerland.

Sephadex G 200 from Pharmacia, Uppsala, Sweden.

Blue Dextran Pharmacia, Uppsala, Sweden, for marking the high molecular weight fraction in gel filtration experiments.

Gel filtration on Sephadex G 200. A Sephadex Laboratory column (Pharmacia, Uppsala, Sweden) diameter 25.4 mm, length 450 mm was used. The height of the Sephadex G 200 column was 400 mm for filtration of 3 ml serum or euglobulin. In the experiments sodium chloride 0.15 M containing Ca^{++} 10^{-4} M or Na_2EDTA 10^{-3} M was used as eluent. Before application sera and euglobulin preparations were dialyzed against the same salt solution as that used for the gel filtration. The flow rate, 9 ml per hour, was regulated by a pump. Fractions of 4.5 ml were collected.

[illegible]

buffer in a concentrati[illegible]n of 5×10^{8} cells per ml.

R reagents, serum or serum fractions in which complement components are selectively removed or inactivated. R4 was prepared according to *Pillemer et al.* (1956). R1 and R2 were prepared according to *Fjellström* (1962).

MgEDTA serum To normal human serum diluted 1/15 in VB was added MgNa$_2$ EDTA to a final concentration of 0 04 M This reagent was used as a source of C 2 C 3 and C 4 Complete lysis occurred on incubation of 0 25 ml of this reagent with 1

0 065 To three rows of tubes containing 1 ml EA$_4$ was added 0 3 ml of the fractions from the Sephadex G 200 filtration The tubes were incubated in an icebath for 20 minutes centrifuged and washed once in the VB sucrose The cells in the tubes in one row were resuspended in 1 ml of the VB sucrose and 0 25 ml MgEDTA serum (final concentration MgEDTA in the tubes = 0 008 M) was added After incubation of the tubes at 37° C for 60 minutes the degree of lysis was determined and taken as a measure of the content of C 1a in the fractions For C 1 estimation the cells of the other two rows of tubes were resuspended in VB sucrose ionic strength 0 065 and the tubes were incubated further at 37° C for 60 minutes The tubes were centrifuged and the cells suspended in 1 ml of VB sucrose To the one row was then added 0 25 ml MgEDTA serum and to the other 0 25 ml veronal buffer containing 0 04 M Na$_2$MgEDTA for control purposes The degree of lysis recorded in the tubes with added MgEDTA serum after incubation at 37° C for 60 minutes was taken as a measure of the content of C 1 in the fractions

Fractions containing EDTA were recalcified before testing

C 4 destroying activity Normal human serum heated at 56° C for 10 minutes was used as a source of C 4 On dilution 1/16 0 1 ml of this heated serum showed complete lysis of 1 ml EA in VB with 0 1 ml of an R4 reagent in a final volume of 1 3 ml and no lysis in combination with R1 and R2 reagents To tubes containing 0 1 ml of the C 4 was added 0 1 ml of various dilutions of the fractions obtained by gel filtration After incubation at 37° C for 20 minutes 1 ml EA in VB and 0 1 ml R4 were added to the tubes The hemolysis was recorded after incubation at 37° C for 30 minutes

ATEe hydrolyzing activity was determined with a pH stat apparatus (Radiometer Copenhagen) as described by *Laurell et al* (1965) with the exception that the phosphate buffer used was 0 005 M Enzyme activity was expressed in units according to *Levy & Lepow* (1959)

Determination of C 1 esterase inhibitor To an activated euglobulin as C 1 esterase source in the above mentioned C 1 esterase test was added 0 25 0 2 or 0 1 ml of various fractions to test C 1 esterase inhibitor The influence on reaction rate was determined The amount of inhibitor was expressed in units according to *Levy & Lepow* (1959)

RESULTS

Gel filtration on Sephadex G 200 was performed with pooled normal human serum activated euglobulin HANE serum and purified preparations of C 1 esterase (activated C 1s) Before filtration the sera and the euglobulin preparations were divided into two parts One part was dialyzed against 0 15 M sodium chloride containing 10^{-4} M Ca^{++} and one against 0 15 M sodium chloride containing 10^{-3} M sodium EDTA The Sephadex G 200 columns were equilibrated and elution was performed with the same salt solution as that used for dialysis The fractions obtained after gel filtration were studied for C 1 and C 1a and for capacity of inactivating C 4 and hydrolyze ATEe Fractions obtained after gel filtration in the presence of EDTA were recalcified before tested on C 1 and C 1a C 1 esterase inhibitor was localized in the experiments with whole normal serum

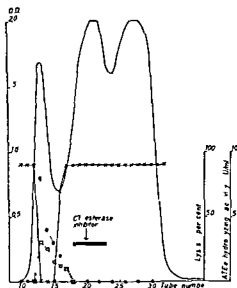


Fig 1

Gel filtration on Sephadex G 200 of normal human serum
Eluent saline containing Ca^{++}

——— O D 280 mμ
○ ○ ○ C̄1a
×—×—× C 4 destroying activity Fractions tested undiluted
□ □ □ ATEe hydrolyzing activity

A *Normal Human Serum*

Gel filtration of normal human serum in saline containing Ca^{++} (Fig 1) resulted in C 1a C 4 destroying and ATEe hydrolyzing activities in the 19S peak

No C 4 destroying ATEe hydrolyzing or C 1 activities were found in the recalcified fractions obtained after gel filtration of normal serum in the presence of EDTA

B *Activated Human Euglobulin Preparations*

On gel filtration of activated euglobulin in saline containing Ca^{++} C 4 destroying and ATEe hydrolyzing activities were found in the 19S peak Also C 1a appeared in the same fractions (Fig 2 A)

After gel filtration with saline containing EDTA C 4 destroying activity was found over a broad region (tubes 15–30) The highest activity was recovered from fractions belonging to the slower part of the second peak (Fig 2 B), where also ATEe hydrolyzing activity and C 1a was found The same results were obtained when the gel filtration was performed with triethanolamine buffer containing EDTA as eluent and with the euglobulin dissolved in this same buffer

The active fractions of the 7S peak from three filtration experiments

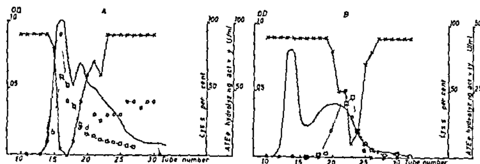


Fig 2

A Gel filtration on Sephadex G 200 of activated euglobulin Eluent triethanolamine buffer containing Ca^{++} *B* Gel filtration on Sephadex G 200 of activated euglobulin Eluent triethanolamine buffer containing EDTA

———— O D 280 mμ
O O O C'1a
×—×—× C'4 destroying activity Fractions diluted 1/80 in VB
□ - □ - □ ATEe hydrolyzing activity

with EDTA were pooled, dialyzed against sodium chloride containing Ca^{++}, concentrated 20 times and refiltrated in the presence of Ca^{++}. C'4 destroying activity was found over a broad region (tubes 15–30) with the highest activity in fractions 23–25, corresponding to the slower part of the 7S peak of the chromatogram of whole serum or euglobulin C'1a was recovered from fractions appearing earlier in the chromatogram, fractions 17–25 (Fig 3) The peaks of C'4 destroying activity and of C'1a therefore appeared in separate fractions No activity could be detected in the fractions corresponding to the 19S peak of whole serum or euglobulin chromatograms

In other experiments all the fractions obtained by gel filtration of euglobulin in the presence of EDTA were pooled, dialyzed against sodium chloride containing calcium, concentrated, and gel filtration was repeated in the presence of calcium C'4 destroying activity and C'1a now appeared in the 19S peak (Fig 4)

C *HANE Serum*

Gel filtration of HANE serum was performed with sodium chloride containing Ca^{++} as eluent (Fig 5A) C'4 destroying activity was found throughout the fractions when tested undiluted The test with diluted fractions revealed that the C'4 destroying activity was localized to the 19S peak ATEe hydrolyzing activity and C'1a were also localized to the fractions in the first peak

On gel filtration of HANE serum in the presence of EDTA the C'4 destroying and ATEe hydrolyzing activities were localized mainly to fractions in the slower part of the second peak, but activity was also found in the 19S peak (Fig 5b) No hemolytic C'1 activity could be demonstrated in any of the fractions

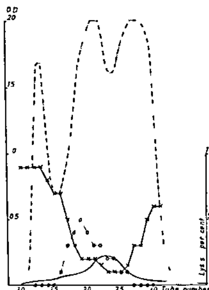


Fig 3

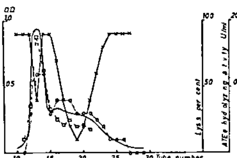


Fig 4

Fig 3 Refiltration of recalcified C'1a containing fractions from the second peak of chromatograms of activated euglobulin (see Fig 2 B) Eluent saline containing Ca^{++}

——————— O D 280 mμ
O O O C 1 a
×—×—× C 4 destroying activity Fractions diluted 1/10 in VB
– – – – Protein curve regularly obtained on filtration of normal human serum (for localization of the protein peak of the refiltrated material)

Fig 4 Refiltration of all the protein containing fractions obtained after gel filtration with EDTA of activated euglobulin These frections were recalcified and concentrated before refiltration Eluent saline containing Ca^{++}

——————— O D 280 mμ
O O - O C 1 a
×—×—× C 4 destroying activity Fractions diluted 1/10 in VB
□ - □ - □ ATEe hydrolyzing activity

D *Partially Purified C'1 Esterase (Activated C'1s)*

was dialyzed against 0 15 M sodium chloride containing 10^{-4} M Ca^{++} and gel filtration was performed with the same salt solution as eluent In order to locate the first and third peak of the gel filtration chromatogram, 0 1 per cent Dextran blue and 0 1 g highly purified albumin was added to 3 ml of C'1 esterase preparation C'4 destroying and ATEe hydrolyzing activities were localized to fractions corresponding to the second peak of a whole serum filtration pattern No C'1 or C'1a could be shown in any of the fractions (Fig 6)

[1] Human albumin kindly supplied by AB KABI Stockholm

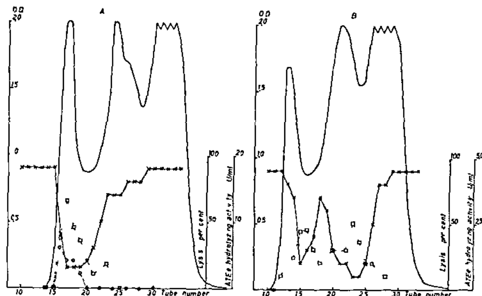


Fig 5

Gel filtration on Sephadex G 200 of HANE serum *A* Eluent saline containing Ca^{++} *B* Eluent saline containing EDTA

————	O D 280 mμ
○ ○ ○	C'1a
×—×—×	C'4 destroying activity Fractions diluted 1/40
□ □ - □	ATEe hydrolyzing activity

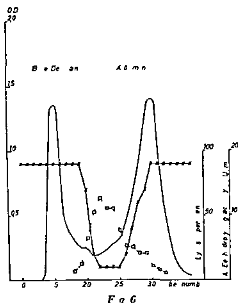
Localization of C'1 Esterase Inhibitor

Low inhibitor content (0 5–1 U/ml was found in the fractions of the second peak after gel filtration on Sephadex G-200 of normal serum (Fig 1) After concentration of the fractions of the second peak 6–7 times in two pools the inhibitor content of the pool comprising tubes 19–21 was 10 6 U/ml and that comprising tubes 22–25 2 6 U/ml

DISCUSSION

Human C'1 is a macromolecule with a sedimentation constant of 18-19S (*Naff et al* 1964, *Borsos & Rapp*, 1965) The present investigation shows that the 19S fraction obtained after gel filtration of normal human serum, euglobulin or HANE serum on Sephadex G 200 in sodium chloride containing Ca^{++} shows C'1a, C'4 destroying and ATEe hydrolyzing activities

After gel filtration of euglobulin and of HANE serum in the presence of EDTA C'4 destroying and ATEe hydrolyzing activities were localized to the slower part of the second peak, and with euglobulin also C'1a was found in these fractions of the second peak No C'1a or C'1 could be shown in the fractions obtained with HANE serum This might possibly depend on storage of the HANE serum resulting in inactivation



F g 6

Cel filtrat on on Sephadex G 200 of pui f ed C 1 esterase Eluent sal ne conta n ng Ca Blue dextran an l all umin \ ere added to the C 1 esterase preparation l efore f ltration t perm t localizat on of the C 4 destroying actn ty

———— O D 280 mμ
X—X—X C 4 destroying actn ty Fractions d luted 1/?0
□ □ □ ATEe hydrol zing activity

of any of the C 1 subcomponents *Lepow et al* (1963) found that C 1 after removal of Ca showed an excessive heat lal ility which *Lepow et al* (1965) have ascribed to the lability of C 1r

When normal serum was fractionated in the presence of EDTA no C 1 or C 1a appeared in any of the fractions neither could any C 4 destroying or ATEe hydrolyzing activities be demonstrated Judging from the data presented by *Lepow et al* (1963) on fractionation of human euglobulin containing C 1 in the precursor form on DEAE cellulose in the presence of EDTA the C 1 macromolecule split and the subunits were separated When the subunits were recombined in the presence of Ca^{++} C 1 hemolytic activity reappeared It is possible that the subunits C 1q C 1r and C 1s which have been shown to have a sedimentation constant of 11S 7S and about 4S respectively (*Naff et al* 1964) on gel filtration of euglobulin on Sephadex G 200 in the presence of EDTA are separated but overlap in the fractions of the slower part of the second peak resulting in C 1a on recalcification of the fraction It should be observed that the euglobulin was prepared according to the method of *Haines & Lepow* (1964) These preparations are void of C 1 esterase inhibitor or contain only traces of it (*Lepow* 196?) In the present investigation it was shown that the C 1 esterase inhibitor of normal serum after fractionation was localized to those fractions of the

second peak which correspond to the fractions containing C'1a and obtained by gel filtration of euglobulin in the presence of EDTA

The finding of C'1a in the second peak after gel filtration in the presence of EDTA of euglobulin may therefore be explained by C'1q, C'1r and activated C'1s being dissociated but situated in the same fractions The failure to show C'4 destroying or ATEe hydrolyzing activity after fractionation of normal serum in the presence of EDTA could be explained by the findings of *Lepow et al* (1963), which showed the necessity of activation of the intact C'1 macromolecule before the separation in subunits to obtain an active low molecular C'1 esterase (activated C'1s) Also the presence of C'1 esterase inhibitor might mask possibly active C'1s present in the same fractions The fact that no C'1 or C'1a appeared might also be explained by the presence of C'1 esterase inhibitor in the same fractions as the C'1 subcomponents The technique used for demonstration of hemolytically active C'1 is based on a stepwise reaction of EA_1 cells first with C'1 followed by addition of a reagent containing C'2 and C'3 The presence of C'1 esterase inhibitor in the first reaction step may therefore block the reaction between C'1a on the cells and added C'2 (*Leon & Lepow* 1962, *Lepow & Leon* 1962)

Naff et al (1964) found that on recalcification and mixing C'1q C'1r and C'1s fractionated by DEAE cellulose chromatography in the presence of EDTA, complexed to a macromolecule with an estimated sedimentation value of 18S capable of forming EAC'1 cells The finding of hemolytically active C'1 in the recalcified fractions of the second peak and not in the fractions of the first peak after gel filtration of human euglobulin in the presence of EDTA is consistent with the result of *Naff et al* (1964) Under such conditions C'1a splits into C'1q, C'1r and activated C'1s and on gel filtration these components may be eluted in peak II, but with different maxima Overlapping of C'1q, C'1r and C'1s in the fractions may explain C'1 hemolytic activity on recalcification of the fractions Refiltration in the presence of Ca^{++} of these C'1a containing fractions of the second peak after recalcification and concentration (Fig 3), however, resulted in C'4 destroying and ATEe hydrolyzing activity in the slow fractions of the second peak C'1a was also recovered from the fractions corresponding to the second peak, but the C'1a maximum was localized to fractions eluted earlier than those containing the greatest C'4 destroying activity Under these conditions C'1a was expected to be localized to fractions corresponding to the 19S peak (*Naff et al* 1964), but it was not it was instead found in fractions corresponding to the more rapid part of the second peak This might mean that C'1q, C'1r and activated C'1s present in the concentrated, recalcified material form a complex with C'1a activity and with a molecular size smaller than the protein eluted in the 19S peak The findings also indicate that after refiltration fractions are obtained containing a surplus of activated C'1s which has not complexed with C'1q and C'1r present in the starting material

In contrast refiltration in the presence of Ca^{++} of a recalcified, concentrated mixture of all the fractions obtained after gel filtration of the euglobulin in the presence of EDTA resulted in C'1a and C'4 destroying activity, but now, in the 19S peak.

These findings indicate that a further factor besides C'1q, C'1r and C'1s might be associated with the native C'1 macromolecule in serum, a possibility which has been proposed by *Naff et al.* (1964). Or, in the mixture of the active fractions of the experiment in Fig. 3 there might be a disproportion between C'1q, C'1r and activated C'1s, which on recalcification does not result in the formation of native 19S C'1a but in a complex with lower molecular weight but with hemolytic activity.

After gel filtration in the presence of Ca^{++} of purified activated C'1s, C'4 destroying and ATEe hydrolyzing activities were recovered from fractions corresponding to the second peak, *i.e.* findings differing from those obtained when normal serum, HANE serum and normal euglobulin were fractionated under the same conditions. During the purification procedure of C'1 esterase according to *Haines & Lepow* (1964) C'1a is apparently split into its subunits, and activated C'1s, C'1 esterase, is prepared free from C'1q and C'1r.

Lepow et al. (1963) claimed that on activation of C'1 to C'1 esterase the hemolytic activity of C'1 disappeared. In the present investigation hemolytically active C'1, C'1a, appeared in the 19S fractions as did C'4 destroying and ATEe hydrolyzing activities, when normal serum, euglobulin or HANE serum were fractionated in the presence of Ca^{++}. *Borsos et al.* (1964) have shown that C'1 in the precursor state reacts with EAC'4 cells to form EAC'14. These EAC'14 cells cannot convert C'2 to C'2a. After incubation at 37° C washed EAC'14 cells are converted to EAC'1a4 cells, which are capable of converting C'2 to C'2a. Partial purification of guinea pig C'1 by fractionation on DEAE cellulose (*Borsos & Rapp* 1963) resulted in a product of C'1a. But purification of human C'1 by the same method resulted in a preparation containing C'1 in the precursor form. The present investigation, however, shows that on gel filtration of human serum fractions are obtained which contain C'1a.

The C'1 esterase inhibitor normally occurring in serum (*Ratnoff & Lepow* 1957, *Levy & Lepow* 1959) inhibits C'1 esterase (C'1a and activated C'1s) but does not inhibit the conversion of C1 to C'1 esterase (C'1a) (*Lepow et al.* 1965). *Lepow et al.* (1965) have given data possibly favouring the assumption of an autocatalytic generation of C'1 esterase (C'1a) in euglobulin, but they also consider the possibility that removal of a second inhibitor permits the conversion of C'1 to C'1a. On fractionation on Sephadex G 200 the C'1 esterase inhibitor, which *Pensky et al.* (1961) showed to be a 4.5 S globulin, separated from the C'1 in the 19S peak. The finding of C'1a, C'4 destroying and ATEe hydrolyzing activities in the 19S peak after gel filtration of normal serum may indicate the possible existence of a factor in serum in-

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PHOTOMETRIC COUNTING OF FORMALINIZED ERYTHROCYTES

By

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Received 28 ix 66

Erythrocytes stabilized by formalinization (7) have replaced fresh red cells in various kinds of hemagglutination reactions (4, 6, 8, 10, 13, 16, 17) Formalinized erythrocytes have so far been quantitated by hemocytometers (9) or by photometric methods with an unknown precision (6, 13) Since the error of the estimate of red cell count by hemocytometers is considerable (2), a rapid and more precise method without employment of expensive and highly spesialized instruments such as electronic cell counters (3, 20) would be desirable

In previous photometric studies (6, 13), formalinized red cells have been quantitated apparently without examinations on the absorption spectrum of such cells In order to obtain maximal sensitivity of photometric determinations, these should be performed at wavelengths giving absorption maxima of the absorbing material The present report describes a photometric method for estimation of tanned or un-tanned formalinized erythrocytes based on their absorption spectrum

MATERIALS AND METHODS

Erythrocytes Human O blood collected on "ACD" medium, was used immediately after the bleeding

[illegible]

vative

Tannic acid Digallic acid was obtained from Matheson Coleman & Bell, Norwood Ohio U S A., and dissolved to 1 40 000 (0 000078 M) by PBS 7 2 immediately before use

Formaldehyde Undiluted Sol formaldehydi (approximately 40 per cent) was used for formalinization

Preparation of formalinized human erythrocytes (FHF) The method of *Csizmas* (5) was employed as elaborated by *Tonjum* (19) Human erythrocytes in 'ACD' medium were washed 7 times in 10 volumes of PBS 6 9 each time by centrifugation at 400 × g for 10 minutes and finally resuspended to a concentration of 12 5 per cent packed cells in PBS 6 9

Formaldehyde was added through a cellophane dialysis bag which contained a volume equal to 1 4 of the erythrocyte suspension In this way the red cells were gently exposed to formaline and left for 20 hours at room temperature under con

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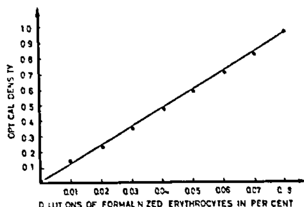


Fig 2

Optical density related to the concentration of formalinized human erythrocytes at concentrations varying from 0.01–0.08 per cent

followed *Beer's* law (1) which states that O.D. are proportionally related to the concentration of the light absorbing material. FHE were diluted to concentrations ranging from 0.01 per cent to 0.08 per cent and their corresponding O.D. recorded on the basis of duplicate determinations. In Fig. 2 O.D. are plotted against the different concentrations. The line was derived by the method of the least squares according to *Kemp & Nielsen* (15). The coefficient of correlation was calculated to $r = 0.9996$. The probability that this correlation should not be linear is very close to zero ($t = 86.6$). Consequently, light absorption of FHE at 4120 Å seems to follow Beer's law in the investigated concentrations.

The Precision of the Method

30 samples were prepared by dilution 1:100 with volumetric pipettes from a solution containing 3 per cent FHE. The O.D. of each sample was recorded on the basis of double determinations with a solution of O.D. = 0.350 as standard reference. In attempts to avoid bias due to conscious or unconscious equalization 10 samples with unknown and varying cell concentrations were mixed with the other 30 in an unknown pattern coded by a collaborator. The results may be summarized as follows:

Number of observations	$n = 30$
Range of observations	0.330–0.360
Mean	$\bar{x} = 0.343$
Standard deviation of the distribution	$s = 0.0052$
Coefficient of variation	$V = 1.5$ per cent

Furthermore, 10 samples were prepared by photometric determinations to a concentration of O.D. = 0.350. The red cell concentrations of these samples were determined by means of a Celloscope which counts 0.8 ml. Ten parallel dilutions of 1:40 were counted from one

sample as well as one dilution from each of the 10 samples In order to eliminate variation due to the Celloscope itself, each dilution was determined by 5 parallel counts The results obtained may be summarized as follows

A 10 observations from one sample	n = 10
Range of observations	473 490
Mean	x = 483
Standard deviation of the distribution	s = 6 7
Coefficient of variation	V = 1 4 per cent
B 1 observation from 10 samples	n = 10
Range of observations	453 500
Mean	x = 479
Standard deviation of the distribution	s = 16.5
Coefficient of variation	V = 3 4 per cent

From the mean of the 10 samples 479 × 10* cells the cell concentration of a solution of O D = 0 350 may be calculated to 479 × 10* × 40 × 1 25 = 2395 × 10³ cells per ml which gives a cell concentration of 239500 cells per mm³ of the original cell suspension

DISCUSSION

The present study demonstrates that tanned and un tanned FHF reveal an absorption band at 4120 Å This band is most likely due to hemoglobin or some of its derivatives *Soret* (18) first observed this band which is the most intense of the hemoglobin bands (12) and which has been applied to determinations of small quantities of hemoglobin (11) Since a standardized control of FHF may be kept for months or years as a control for the spectrophotometer itself there should be no danger of selecting the peak itself for quantitative determinations

Light absorption of FHF at the Soret band followed Beer s law in the investigated concentrations (Fig 2) Consequently the cell concentration of a sample with a known O D may be calculated directly from a reference O D with a determined number of cells per ml

The precision of the method was determined in two ways By the first method the variance of 30 parallel samples which were obtained from the same stock solution was estimated The coefficient of variation was calculated to 1 5 per cent Secondly 10 samples were determined to the same O D and their cell concentrations measured by an electronic counter The coefficient of variation of these 10 samples was calculated to 3 4 per cent while the variation due to dilution and counting itself only was 1 4 per cent Consequently the coefficient of variation of 3 4 per cent mainly reflects variability of the O D determinations (V_{OD} = $\sqrt{3\,4^2 - 1\,4}$ = 3 1 per cent)

The discrepancy of variation between the two methods may be due to bias of the first method because of equalization although attempts

* = cells counted in hundreds

were made to avoid this The last method, therefore, probably gives a more correct picture of the precision of the method which seems to be in the order of 3 per cent This is more than the variability (coefficient of variation) of the Celloscope found to be 2 per cent by *Brecher et al* (3), but considerably less than the variability of hemocytometers which have been estimated to 7 8 per cent (2) In order to get a coefficient of variation of 3 per cent with hemocytometers, 8 parallel countings have to be undertaken (3)

The present studies referred to the same stock solution of FHE and variation due to individual differences between blood donors or separate formalinization procedures have not been investigated

SUMMARY

A photometric method for the counting of formalinized erythrocytes based on an absorption band at 4120 Å has been described Light absorption at that wavelength by formalinized red cells was subjected to Beer's law in the concentrations investigated

The methodological variability as expressed by the coefficient of variation was determined to be in the order of 3 per cent which is close to that of electric cell counters (2 per cent) and considerably less than the variability of hemocytometers (7 8 per cent)

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ON THE SORPTION OF THYROGLOBULIN TO FORMALINIZED AND TANNED HUMAN ERYTHROCYTES

By

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A variety of antigenic substances may be attached to the surface of red cells While some polysaccharides combine with the red cell directly (11) proteins usually require a previous treatment of the erythrocyte by certain substances such as tannic acid (1)

In general molecules may be connected to a surface by simple physical adsorption or by bonds of chemical nature These two types of attachment differ in several respects, one of which is their relationship to temperature While the quantities of physically adsorbed material increases with decreasing temperatures substances requiring chemical bonds are more readily attached when the temperature is increased

Certain polysaccharides seem to be sorbed to red cells by a reaction of chemical nature (10) Less is known about the sorption of protein antigens By using I^{131} labelled human serum albumin *Daniel et al* (2) found increasing quantities attached to red cells at increasing temperatures These results would seem to indicate that the sorption of proteins to tanned red cells also might be of chemical nature

Thyroglobulin is readily sorbed to fresh (13) as well as formalinized tanned erythrocytes (9) and the method of indirect hemagglutination is widely used for detection of thyroglobulin auto antibodies But the nature of the binding by which thyroglobulin is sorbed to tanned erythrocytes remains unknown In the present study the relationship of this binding to temperature was investigated

MATERIALS AND METHODS

addition of merthiolate to a concentration of 0 01 per cent and stored at 4° C until used in a dilution of 1 100 in PBS 7 2

Antigens Human thyroglobulin was prepared from operation specimens of non toxic goitres according to *Derrien et al* (3) as modified by *Roitt & Doniach* (12) The final thyroglobulin solution was lyophilized and stored as powder at —20° C. Bovine thyroglobulin was prepared in an identical manner from bovine thyroids cbt

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a dilution of 1 2500 while serum *U R* gave no cross reaction with bovine thyro globulin The sera were inactivated at 56° C for 10 minutes in a dilution of 2 volumes serum to 7 volumes PBS 7 2 and absorbed at 4° C over night with 1 volume of a 10 per cent suspension of tanned FHF Serum dilutions were made in NRS

Sensitization of FHF The red cells were sensitized after treatment with tannic acid Since preliminary studies did not reveal any temperature to be better than others tanning was performed at room temperature for 15 minutes FHE in a concentration of 240 000 cells per mm³ (O D in dilution 1 100 = 0 350) were exposed to an equal volume of digallic acid Optimal concentrations were found to range from 1 20 000 to 1 80 000 Consequently the red cells were treated with digallic acid at a concentration of 1 40 000 (0 000078M) in PBS 7 2 for 15 minutes at room temperature The cells were then washed twice in PBS 7 2 and resuspended in PBS 7 2 to half the concentration of the initial suspension

These cells were exposed to an equal volume of antigen for 30 minutes and after wards usually washed 3 times with NRS at the same temperature as the treatment with antigen The cells were finally made up to a concentration 1 4 of the original suspension (60 000 cells per mm³)

Hemagglutination reaction Hemagglutination reactions were carried out in Perspex agglutination trays 0 1 ml of each serum dilution and 0 1 ml of sensitized or un sensitized FHE were added to each cup and mixed gently The trays were left at 4° C over night and read according to *Fulthorpe et al* (4)

Hemagglutination inhibition assay The hemagglutination reaction for detection of thyroglobulin antibodies may be reversed and used for detection of thyroglobulin (8) In the present report this method was employed for quantitative measurements of thyroglobulin attached to red cells 0 1 ml of serum dilutions and 0 1 ml of thyro globulin solutions or thyroglobulin sensitized FHF were mixed in test tubes 10 by 80 mm and left at 4° C over night 0 1 ml of the supernatant was then tested for hemagglutination activity as described above

RESULTS

Quantitative Evaluation of Hemagglutination Inhibition

Concentrations of thyroglobulin ranging from 0 01 to 10 microgr per ml were tested in a chequerboard pattern against twofold dilutions of serum U R ranging from 1 250 to 1 64 000 which was the titer ob tained when no antigen was added to the serum In Fig 1 the concen trations of antigen used for absorption are plotted against correspond ing reductions of hemagglutination Although antigenic determinants on the thyroglobulin molecule may be 'hidden" as a consequence of attachment to the red cells, employment of Fig 1 should give a correct picture of the relative quantities of thyroglobulin bound to the red cells

Studies on the Sorption of Thyroglobulin to Tanned FHE at 0° C and 37° C

The quantities of thyroglobulin sorbed to tanned FHE exposed to various concentrations of antigen at 0° C and 37° C are shown in Fig 2

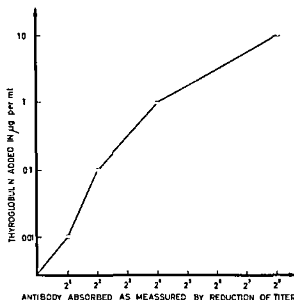


Fig 1

Hemagglutination inhibition of human thyroglobulin auto antibodies (serum U R) by different concentrations of human thyroglobulin

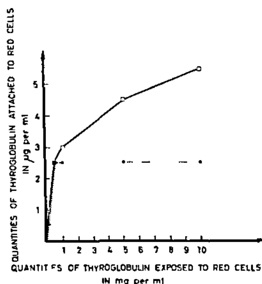


Fig 2

Uptake of human thyroglobulin by tanned formalinized human erythrocytes exposed to various concentrations of thyroglobulin at 0° C (□—□—□) and 37° C (○ – ○ – ○)

Each point was estimated from Fig 1 on the basis of 4 parallel experiments

As may be seen from Fig 2 approximately only one of thousand molecules was attached to the cells Cells treated with antigen concentrations up to 0 5 mg per ml sorbed similar amounts at 0° C and 37° C At higher concentrations of antigen however larger quantities were attached at 0° C than at 37° C Furthermore whereas the quantities bound at 0° C increased with increasing quantities of thyroglobulin added up to 5 5 microgr per ml by cells exposed to 10 mg per ml no increased uptake could be detected at 37° C by cells exposed to thyroglobulin concentrations varying from 0 5 to 10 mg per ml

Cells treated with 10 mg per ml at 0° C could be washed 8 times at 0° C without loss of antigen But if these cells were incubated at 37° C for 30 minutes the concentration of cell bound antigen decreased to 2 5 microgr per ml and free thyroglobulin could be detected in the supernatant (0 5 2 5 microgr per ml) after sedimentation of the cells

The specificity of the reaction was controlled by using bovine thyroglobulin By employment of serum S A bovine thyroglobulin also revealed increased attachment to cells exposed to 5 and 10 mg per ml at 0° C as compared to 37° C These cells did however not absorb any detectable quantities of antibody from serum U R

Attempts were made to study the kinetics of the sorption of thyroglobulin to tanned FHE but even after 5 minutes maximal amounts seemed to be bound to the cells at 0° C as well as 37° C Un tanned FHE did not take up any antigen at either 0° C or 37° C

DISCUSSION

From the presented results it may be concluded that larger quantities of thyroglobulin were attached to tanned FHE at 0° C than at 37° C This observation indicates that thyroglobulin may be connected to these cells by a thermolabile binding such as simple physical adsorption Further support for a binding of this type was achieved from studies on the reversibility of the reaction The surplus of antigen sorbed at 0° C was easily liberated by heating to 37° C In both these respects thyroglobulin behaved similarly as cold agglutinins (6)

On the other hand considerable quantities remained bound at 37° C independent of the quantities attached at 0° C and were not removed by repeated washings at 37° C Furthermore FHE exposed to concentrations less than 1 mg per ml of thyroglobulin sorbed approximately the same amounts at 37° C as compared to 0° C These observations are difficult to explain simply on the basis of physical adsorption and seem to indicate that a binding stabile at 37° C also is involved in the attachment of thyroglobulin to tanned FHE The stability at 37° C would suggest that this binding may be of chemical nature The sites available on the cell surface for the latter type of binding seem to be

limited because no additional antigen was sorbed when the cells were exposed to quantities of thyroglobulin varying from 0 5 to 10 mg per ml

Since the velocity of both reactions apparently was very high, either of them probably participates in the sorption at 0° C This may explain why only excess of thyroglobulin sorbed at 0° C as compared to 37° C were liberated by heating to 37° C

The present results are at variance with earlier studies *Jyssum* (10) investigated the sorption of pneumococcal polysaccharide C to fresh sheep erythrocytes By several methods this binding was established to be a chemical reactions of 1 order *Daniel et al* (2) found increasing amounts of I^{131}–labelled human serum albumin to be connected to tanned and formalinized sheep erythrocytes with increasing temperatures Their results indicate that at least a majority of human serum albumin molecules are sorbed to tanned red cells by a reaction of chemical nature

On the basis of the present studies, thyroglobulin seems to be at tached to tanned red cells by bindings of both chemical and physical nature Why thyroglobulin in this respect behaved differently than other molecules remains unknown Thyroglobulin has a large molecular weight (700 000) (7), and antigenic uptake of red cells by simple physical bindings may be peculiar to such giant molecules

SUMMARY

The sorption of human thyroglobulin to tanned and formalinized human erythrocytes has been investigated by means of the hem agglutination inhibition reaction Thyroglobulin was readily attached to these cells at 0° C as well as 37° C Cells exposed to antigenic concentrations of 1–10 mg per ml took up larger quantities at 0° C than at 37° C, although considerable amounts were firmly attached at the latter temperature The excess quantities of thyroglobulin bound at 0° C could be liberated by heating to 37° C These observations were interpreted as showing thyroglobulin to be connected to red cells both by a thermolabile binding of physical nature and a thermostabile binding probably chemical in nature

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QUANTITATIVE EXAMINATION OF AGGLUTINATION AND PROZONE FORMATION OF THYROGLOBULIN COATED ERYTHROCYTES BY HUMAN AUTO ANTIBODIES

By

TORF GODAL

Received 28 iv 66

The indirect hemagglutination reaction may be divided into 3 steps

1 Attachment of antigen to red cells
2 Specific interaction between antigen and antibody
3 Aggregation of red cells (the hemagglutination itself)

In a previous study (4) the first and second step were isolated from the third by use of the hemagglutination inhibition reaction By this method the sorption of thyroglobulin to red cells could be investigated by antibody absorption of such cells

The third step of the indirect hemagglutination reaction seems to be due to the formation of cross linkages between sensitized erythrocytes formed by bivalent antibody molecules (7) This reaction may be inhibited by excess of antibody (5 13) and by certain "blocking anti bodies (14) giving rise to the so called prozone phenomenon (11) or by univalent antibody fragments (7)

Less is known about the influence of the antigenic determinants attached to the red cells on the resulting agglutination Certainly a minimum of antigenic determinants are required in order to get agg lutination (10) but *Daniel et al* (1) also reported decreased agglutin ation when large amounts of antigen was connected to the cells Since the antigenic uptake of the cells was determined by radioactivity it remains unknown wether this inhibition affected the second or the third step of hemagglutination

Although the indirect hemagglutination procedure for detection of thyroglobulin antibodies has been established in several laboratories information about the correlation between the quantities of thyroglob ulin sorbed to the cells and hemagglutination and prozone patterns are lacking Furthermore the concentrations of antigen employed for sen sitization of red cells vary (2 9 12 15) The purpose of this study was

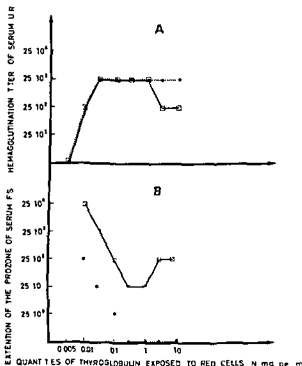


Fig 1

Agglutinability (Fig 1 A) and prozone formation (Fig 1 B) of tanned formalinized human erythrocytes exposed to various concentrations of human thyroglobulin at 0° C (□—□—□) and 37° C (O O—O) by human auto antibodies

to gather information in general about the influence of the antigen concentrations attached to red cells on the final step of the indirect hemagglutination reaction and to define optimal conditions for agglutination of thyroglobulin coated erythrocytes

MATERIALS AND METHODS

The materials and methods employed in the indirect hemagglutination reaction and the indirect hemagglutination inhibition reaction have been described (3 4) Serum U R (4) was used for investigations on agglutination patterns The prozone phenomenon was investigated by serum F S originally generously provided by Dr L Kornstad Serum F S was obtained from a patient suffering of thyroiditis revealing a hemagglutination titer of 2 5 × 10^6 towards thyroglobulin and gave rise to a so called negative precipitin line in gel precipitation as has been described by *Goudie et al* (6) Serum F S produced the most marked prozone phenomenon of the sera investigated in preliminary studies

RESULTS

The resulting hemagglutination titers with serum U R after sensitization of FHE with various amounts of antigen at 0° C and 37° C are shown in Fig 1 A No agglutination appeared with cells exposed to concentrations of thyroglobulin less than 0 01 mg per ml Concentrat

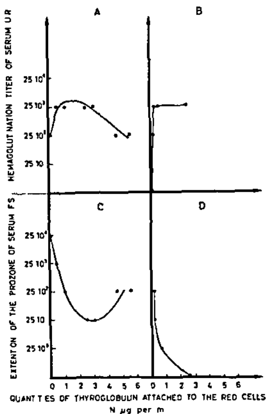


Fig 2

Effect of the concentration of human thyroglobulin attached to tanned formalinized human erythrocytes at 0° C (Fig 2 A and C) and 37° C (Fig 2 B and D) on agglutinability (Fig 2 A and B) and prozone formation (Fig 2 C and D) by human auto antibodies

ions of 0 01 0 05 mg per ml gave increasing hemagglutination titres at 0° C as well as 37° C No further increase of sensitivity of the hemagglutination reaction could be observed by increase of the thyroglobulin concentration In contrast cells exposed to 5–10 mg per ml at 0° C revealed a reduced agglutinability

The corresponding extention of the prozone of serum F S is shown in Fig 1 B The prozone of cells treated at 0° C in general extended longer than at 37° C At concentrations of thyroglobulin less than 0 5 mg per ml at both 0° C and 37° C the prozone was inversely related to the concentration of antigen By using 0 5 mg per ml or higher concentrations of thyroglobulin, the prozone could be completely eliminated at 37° C whereas cells sensitized at 0° C never reduced the prozone to less than 1 250 and revealed an extention of the prozone at antigenic concentrations of 5 10 mg per ml

The correlations between hemagglutination and prozone and the

quantities of thyroglobulin bound to the erythrocytes (4) are shown in Fig. 2. Optimal conditions for agglutination at 0° C were found when 0.5–3 microgr. per ml were attached to the cells (Fig. 2 A). At lower or higher concentrations a reduction of agglutination occurred. A minimal prozone was observed at concentrations of 2.5 3 microgr. per ml (Fig. 2 C). Lower or higher concentrations of antigen extended the prozone. *At 37° C maximal agglutination was obtained with cells containing 0.15 2.5 microgr. per ml (Fig. 2 B).* The agglutinability was reduced at lower concentrations while 2.5 microgr. per ml is the largest amount of thyroglobulin taken up by tanned erythrocytes at 37° C (4). *Cells treated at 37° C* revealed a reduced prozone as compared to cells coated at 0° C although they contained as much or even less thyroglobulin per cell (Fig. 2 C and D). With cells coated at 37° C containing 2.5 microgr. per ml the prozone was completely eliminated (< 1 : 25).

The specificity of the inhibition of agglutination at antigen excess was controlled by bovine thyroglobulin. Cells exposed primarily to 0.5 mg per ml at 37° C and afterwards to 10 mg per ml at 0° C of bovine thyroglobulin *revealed no decreased agglutinability* although bovine thyroglobulin is sorbed to red cells by the same mechanisms as human thyroglobulin (4). Since addition of free thyroglobulin reduced the prozone in the same way as the agglutination reaction free unattached thyroglobulin cannot explain the increased prozone obtained by cell *exposed to large quantities of thyroglobulin at 0° C.*

DISCUSSION

The present investigation would seem to indicate that the concentration of antigen affects the hemagglutination reaction in a way similar to the precipitin reaction. In order to get agglutination a certain minimum of antigenic determinants per red cell was required. When less *than 0.1 microgr.* was *attached to about 6 × 10⁷ cells no agglutination* occurred. Since the molecular weight of thyroglobulin is about 700 000 (8) the minimal number of antigenic molecules required per cell for agglutination should be in the order of

$$6 \times 10^{3} \times 10^{7} \times 1/7 \times 10^{5} \times 1/6 \times 10 \approx 1500$$

Optimal conditions for agglutination with a complete elimination of the prozone was obtained by exposing cells to 0.5 mg per ml at *37° C.* Such cells contained 2.5 × 10⁸ *microgr.* of thyroglobulin or about 35 000 antigen molecules per cell.

These values are lower than estimations from other antigen *antibody* systems as reported by *Luderitz et al.* (10). These authors found the minimal number required for agglutination to be 5 000 molecules *per cell and 100 000 molecules per cell for optimal* agglutinability. The report of *Daniel et al.* (1) also seems to indicate higher values than 35 000 for optimal agglutinability. Since in both *these investigations*

the quantities of antigen attached to the cell were measured by the attachment of radionated antigens labelled but immunologically in active molecules may tend to give too high values On the other hand as earlier reported (4) estimations based on the hemagglutination in hibition reaction may give too small values because antigenic deter minants may be hidden by the attachment of antigen to the cell surface Consequently the difference between the present estimations and earlier reports may be explained on a methodological basis

Since impurities of antigenic preparations increasingly may be sorbed when the antigen concentration is raised cells exposed to larger quan tities than required may give rise to less specific reactions Conse quently for later routine work 0 5 mg per ml of thyroglobulin were selected for sensitization

In contrast to cells sensitized at 37° C cells exposed to antigen at 0° C may take up larger quantities of thyroglobulin than 35 000 mole cules per cell (4) Such cells which contained about 60 80 000 molecules per cell revealed a reduced agglutinability This reduction could not be produced by exposure of bovine thyroglobulin at identical conditions to cells previously coated with human thyroglobulin in optimal con centrations Furthermore while addition of free thyroglobulin reduced the prozone cells exposed to large quantities of thyroglobulin at 0° C revealed an extention of the prozone These observations represent evidence against a reduction of agglutinability of these cells due to a non specific change of the red cells or free thyroglobulin in the solution

Since such cells absorb larger quantities of antibodies the reduced agglutinability must be related to the third step of agglutination Consequently when the antigen concentration on the cell surface in creases above certain levels the cross linkage formation between such cells by homologous antibodies seems to be inhibited In other words hemagglutination reactions seem in accordance with other immuno logical reactions to be inhibited in antigenic excess

In the present studies at 0° C the prozone phenomenon was reduced to a minimum corresponding to optimal concentrations for agglutina tion and was extended at both higher and lower concentrations of antigen on the red cell (Fig 2 C) Increase of the prozone at low anti genic concentrations may easily be explained both by competetive inhibition by blocking antibodies (14) and by excess of antibodies (13) However extention of the prozone at high concentrations of antigen on the cell surface is difficult to explain on the basis of blocking anti bodies Such antibodies should have a reduced opportunity to block at antigenic excess Since *Goodman & Masaitis* (5) found reduced agglutination to be a general phenomenon at antibody excess the ex planation may be that antigenic excess on the cell and antibody excess in solution are two conditions which do not neutralize each other but add their negative influence on the agglutination reaction

As shown in Fig 2 D, the *prozone of* cells treated at 37° C was reduced by cells containing as much or less antigen than cells exposed to antigen at 0° C (Fig 2 C) This observation would seem to support previous studies (4) which *indicated that thyroglobulin is attached to* the red cell in a different manner at 0° C than at 37° C, and not as favorable for agglutination

Hjort (9) observed a prozone phenomenon in the lowest serum dilution (1 5) of several sera which could be eliminated by exposure of the antigen to 56° C for 30 minutes Since Hjort employed 1 5 mg per *ml of thyroglobulin for sensitization at* 37° C, heat *most likely* produces a qualitative alteration of the antigen *favoring* agglutination and not a quantitative alteration of the sorption of thyroglobulin to the cells Consequently, the prozone is *not only* influenced *by* the quantities *of antigen per* cell, but also by the mechanism of attachment and the structure of the antigen

Why serum F S was particularly *prone to give a prozone* remains unknown *This* serum gave *rise* to a high hemagglutination titer (1 2 5 × 10[6]) and a so called "negative" precipitin line in gel precipitation The antibodies of serum F S may react with very few anti... minants on the thyroglobulin mole... rise to ext... agglutin... ...tigen antibody bonds Another ... be that the antigenic determinants to which the antibodies of serum F S combine, not easily are available or orient *the antibodies in unfavorable directions for agglutination* The dependence of the prozone on the mechanism of sorption would seem to support the latter hypothesis

SUMMARY

The sensitivity of the indirect hemagglutination *reaction for detection of thyroglobulin* antibodies and the formation of prozone of such antibodies have been correl... ...en exposed and ... ...° 0 C and 37° C ... required to get agglutination seemed t... ...out 1400 molecules per cell *Optimal* conditions *for agglutination* with a complete elimination of *prozone* were established when tanned formalinized human erythrocytes at a concentration of 1 2 × 10[8] cells per ml were exposed to an equal volume of 0 5 mg of thyroglobulin per ml at 37° C for 30 minutes These cells were estimated to contain about 35 000 thyroglobulin *molecules per cell* A *further* increase of antigenic determinants which was obtained by sensitization at 0° C gave rise to a reduced agglutinability *This reduction* at antigenic excess was *concluded to be related to the cross* linkage *formation* by antibodies

The present studies on the prozone phenomenon *revealed that* the prozone not only depends *on* the concentration *of* antigenic deter

minants per cell, but also on the temperature at which the antigen is attached to the cell In general, cells treated at 0° C revealed a larger prozone than cells sensitized at 37° C This observation supports previous investigations which provided evidence for the sorption of thyroglobulin to red cells by different mechanisms

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CIRCULATING ANTIBODIES IN EXPERIMENTAL MYCOBACTERIAL INFECTIONS DEMONSTRATED BY IMMUNOFLUORESCENCE

By

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Received 7 x 66

The occurrence of circulating antibodies in mycobacterial infections has been demonstrated by various serological procedures However, remarkably few reports have included the fluorescence antibody test (FAT) among the diagnostic tools Some papers deal with the FAT in identification and classification of mycobacteria *Kirsh & Shepard* (1961) and *Shepard & Kirsh* (1961) examined fragments of disrupted mycobacteria by fluorescence staining *Gillisen* (1963) immunized rabbits with heat killed mycobacteria and obtained some specificity with different types of indirect techniques *Jones et al* (1965) used living culture for immunization and were able to differentiate between M tuberculosis, M kansasii and M phlei by immunofluorescence They demonstrated serological differences within Runyon s Group III strains and were able to distinguish Group III strains from M avium

Using sera from lepromatous patients, *Morris et al* (1961) and *Merklen et al* (1963) demonstrated antibodies against M leprae They proved the presence of cross reactivity between M leprae and M tuberculosis and were able to remove it by absorption

The immunofluorescence technique has been adopted in this laboratory in order to examine the dynamics of antibodies related to infection and their specificity for the homologous mycobacteria This paper reports the course of the FAT in serum from rabbits inoculated with virulent strains of M avium and M bovis

MATERIALS

B 1 the strain of M avium used in Series 1 was isolated from the spleen of a hen and proved to be fully virulent for rabbit and hen

E 4584/64 the strain of M bovis used in Series 2 was the first strain isolated from a patient with untreated tuberculosis

The animals used in the study were male albino rabbits bred at this Institute. Each series consisted of 20 rabbits i e 18 for inoculation and 2 controls all weighing 1900–2100 g at the commencement of the experiment

METHODS

Inoculation Ten day old cultures grown in Dubos liquid medium with Tween 80 were adjusted to an optical density of 60 in the Klett Summerson photoelectric colorimeter It was shown by testing on oleic acid agar plates that the suspensions contained about 10^7 viable units per ml Three groups of rabbits were inoculated intravenously with 10 , 10^5 and 10^3 viable units respectively

Weekly examination The weight of the animals was recorded and 5 ml blood drawn from the marginal ear vein for use in the FAT Mantoux testing was per formed on two animals from each group using 100 TL avian PPD RS 10 for Series 1 and 100 TL human PPD RT 23 for Series 2 The reactions were read after 24 hours

Post mortem examination Autopsy was performed on all animals and the gross lesions described Smears from spleen lung liver and joints were examined by fluorescence microscopy after auramine rhodamine staining (*Benne lsen & Olesen Larsen* 1966)

Fluorescence antibody test (FAT)[1] After collection of the serum phenol and FDTA were added to obtain final concentrations of 0 5 per cent. Serum dilutions were made in 0 5 per cent saline buffered with 1/100 M phosphate pH 7 2

Substrate Cultures of the homologous strains were grown on Lowenstein-Jensen tubes and then suspended in buffered saline A drop of the suspension was placed on a sli le which had been washed previously in acetone After drying the smears of M avium were placed in an oven at 80° for 12 hours for fixation The smears of M bovis were submerged in al solute methanol for 15 minutes and dried overnight in the refrigerator

Staining A drop of the test serum dilution was spread carefully over the smear with a glass rod The slides were then placed in a moist chamber for 30 minutes at room temperature After rinsing the slides in buffered saline for 10 minutes the surplus fluid around the smear was removed by absorption with paper tissue

A drop of diluted anti rabbit globulin sheep globulin conjugated with fluorescein iso thio cyanate (FITC) was spread over the smear The slides were again placed in the moist chamber for 30 minutes and then rinsed in buffered saline for 10 minutes

The smears were allowed to dry completely and then mounted in buffered glycerol (pH 9) with a coverslip rinsed in ether

Microscopical examination The large Carl Zeiss fluorescence microscope was equipped with an ultra dark field condensor Generally the objectives used were apochromate $\times$ 40/n A 1 0 oil and planapochromate $\times$ 100/n.A 1.3 oil both with iris The eyepieces were 12.5 $\times$ kpl The primary/secondary filters used were BG 12 47 LG 5/44 and LG 1/41 BG 38 was generally omitted

Registration The reciprocal of the highest dilution with which definite fluores cence still occurred was notified as the titre of the serum The dilutions used were 1/10 1/31 6 1/100 1/316 and 1/1000

Column chromatography A Pharmacia laboratory column K 25/100 was packed with Sephadex G 200 in buffered saline and fitted with an upward flow adaptor The effective length of the column was 93 cm 2 ml of serum was sucked into the bottom of the column and molecular sieving performed with a flow of 25 ml per hour The eluate passed through a Uvicord (LKB) and then through the 5 ml siphon of an automatically operated fraction collector

RESULTS

Series 1 Rabbits Infected with M avium

Fig 1 shows the results of the FAT for the three groups of animals The points represent the logarithmic means of titres from the time of first detectable antibody response until the death of the first animal in the group However in Group 1 rabbit No 7589 died before and No 7594 immediately after, the first samples were drawn Thus the means are

[1] I am indebted to Dr *H Aa Nielsen* Treponematosis Department Statens Serum Institut for help in compiling the technique

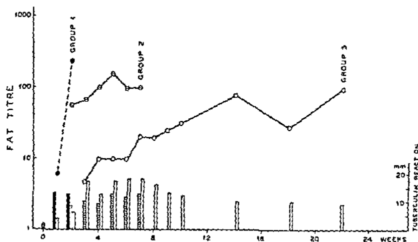


Fig 1

Series 1: Rabbits infected with *M avium*

Curves. Titres of FAT against homologous strain

Columns Results of Mantoux testing with 100 T U avian PPD (RS 10)

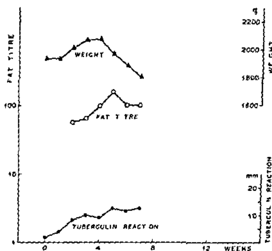


Fig 2

Series 1, Group 2 Rabbits infected with 10^5 viable units *M avium*

calculated from five and four results, respectively In Groups 1 and 3, some sera showed no reaction at the time at which the curves commenced (see Table 1) In order that these should not be excluded, they were given the titre 10^0. The slope of the titre curves and the time of first response seemed to be related to the infection dose, but the final level of antibody was generally the same for all groups The tuberculin reactions are shown in the lower part of the figure Each column represents the arithmetic mean of two reactions of two animals There is no correlation between these results and the titre curves

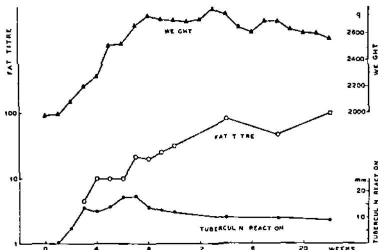


Fig 3
Series 1 Group 3 Rabbits infected with 10^3 viable units M avium

Figs 2 and 3 show the course of infection in Groups 2 and 3, judged by the mean weights of the animals, the fluorescence antibody titres and the tuberculin reactions

Table 1 shows some of the results with the individual rabbits There is some variation in the fluorescence antibody results, but, except for the two animals mentioned above, all the rabbits had maximum titres of 100 or more in their sera during the course of the infection There were no differences in the antibody titres of the animals subjected to Mantoux testing and those not tested

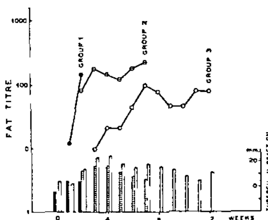


Fig 4
Series 2 Rabbits infected with M bovis
Curves Titres of FAT against homologous strain
Columns Results of Mantoux testing with 100 T U human PPD (RT 23)

TABLE 1

Series 1 Rabbits Infected with M avium

Group	Rabbit	Infection dose (v u)	Survival time (days)	Time of first pos FAT (weeks)	Titre of max FAT	Time of max FAT (weeks)	Titre of term FAT	Acid fast rods in organs	Acid fast rods in joints	Terminal weight (g)
1	7589	10^7	7	n e	n e	n e	n e	+	0	1850
	7590	10^7	18	2	316	2	316	+	+	2010
	7591	10^7	16	1	316	2	316	+	+	1850
	7592	10^7	17	1	316	2	316	+	+	1990
	7593	10^7	21	1	100	2	100	+	+	2020
	7594	10^7	7	1	10	1	10	+	0	1860
2	7595	10^5	55	2	316	5 7	316	+	+	1850
	7596	10^5	121	2	100	2 10	10	+	+	1210
	7597	10^5	188	2	1000	26	1000	+	+	1460
	7598	10^5	111	2	100	2 7	10	+	+	1280
	7599	10^5	73	2	316	5	31 6	+	+	1390
	7600	10^5	72	2	100	2 3	10	+	+	1330
3	7601	10^3	156	3	100	14	31 6	+	+	1510
	7602	10^3	241	3	1000	26	1000	0	+	2510
	7603	10^3	176	4	100	26	100	+	+	2810
	7604	10^3	274	4	100	14	31 6	0	+	1760
	7605	10^3	293	3	316	22	100	0	+	2260
	7606	10^3	241	3	316	22	316	+	+	2080

n e = not examined v u = viable units

TABLE 2

Series 2 Rabbits Infected with M bovis

Group	Rabbit	Infection dose (v u)	Survival time (days)	Time of first pos IAT (weeks)	Titre of max IAT	Time of max IAT (weeks)	Titre of term IAT	Acid fast rods in organs	Terminal weight (g)
1	7657	10^7	24	1	316	3	316	+	1950
	7658	10^7	20	1	100	2	100	+	1700
	7659	10^7	21	1	316	2	316	+	1600
	7660	10^7	26	1	316	2	316	+	2020
	7661	10^7	27	2	316	3	316	+	2040
	7662	10^7	27	1	100	2	100	+	2170
2	7663	10^5	52	2	316	3 8	316	+	1700
	7664	10^5	51	2	316	6 7	316	+	1750
	7665	10^5	99	2	316	7 14	316	+	1630
	7666	10^5	55	2	100	2 7	100	+	1780
	7667	10^5	62	2	100	6	100	+	1950
	7668	10^5	69	2	316	3 8	316	+	2110
3	7669	10^3	116	3	100	6 12	31 6	+	1920
	7670	10^3	111	3	316	12	100	+	1900
	7671	10^3	84	3	100	7 12	100	+	2030
	7672	10^3	96	4	100	7 8	31 6	+	1930
	7673	10^3	86	3	100	6 12	100	+	1500
	7674	10^3	93	3	100	7 12	100	+	1600

v u — viable units

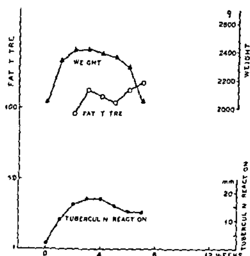


Fig 5
Series 2 Group 2 Rabbits infected with 10⁵ viable units M bovis

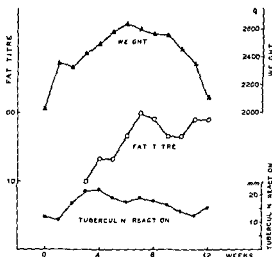


Fig 6
Series 2 Group 2 Rabbits infected with 10³ viable units M bovis

Series 2 Rabbits infected with M bovis

Figs 4 5 and 6 and Table 2 show the FAT results in Series 2 With the exception of a shorter course in Group 3, the results are essentially the same as in Series 1

Serum fractionation *In order to obtain further characterization* of the antibodies demonstrated some serum samples were subjected to Sephadex molecular sieving Fig 7 shows the Uvicord transmission curve for a normal rabbit serum, and superimposed on it the curve for an animal belonging to Series 1, Group 1 Three main peaks were ob

TABLE 3

Fluorescence Antibody Titres of Individual Serum Fractions Obtained by Molecular Sieving through Sephadex G 200
(For details, see text)

Series	Group	Infection dose (v u)	Time of bleeding (weeks)	Fluorescence antibody test (FAT) Titre of total serum	Titre of peak 1	Titre of peak 2	Titre of peak 3	Remarks
1 (Rabbits infected with *M avium*)	1	10^7	2	316	100	100	0	Pool of 4 rabbits
	2	10^5	4	100	31.6	31.6	0	Pool of 4 rabbits
	3	10^3	22	1000	0	316	0	Single serum
2 (Rabbits infected with *M bovis*)	1	10^7	2	316	100	316	0	Single serum
	2	10^5	4	316	0	316	0	Single serum
	3	10^3	12	316	0	316	0	Single serum

0 — no reaction　　v u — viable units

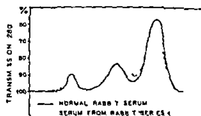


Fig 7

Uvicord transmission curves of serum samples subjected to Sephadex G 200 fractionation

served Table 3 gives details concerning the fractionated sera In both series, the "early" sera (from Group 1) contained antibodies in the first and second peak, in contrast to the 'late' sera (from Group 3) where all the reactivity was found in the second peak In calculating the titres, the 2 ml of serum applied to the column was considered to be diluted to the volume of the pooled fractions corresponding to a peak

TABLE 4

Fluorescence Antibody Test with different Mycobacteria as Substrate

(Serum dilution 1 100 titre for all sera against homologous strain 316)

Substrate	Serum: Series 1 anti M avium (B 1) Group 1 (2 weeks)	Serum: Series 1 anti M avium (B 1) Group 2 (12 weeks)	Serum: Series 2 anti M bovis Group 1 (3 weeks)	Serum: Series 2 anti M bovis Group 2 (8 weeks)
M tuberculosis	—	+	+	+
M tuberculosis	—	—	+	+
M tuberculosis	—	+	+	+
M bovis		+	++	++
BCG	—	—	+	+
M avium	—	—	—	—
M avium (B 1)	++	++	—	—
Photochromogen	—	—	—	—
M aquae	—	—	—	—
M scrofulaceum	—	—	—	—
M phlei	—	—	—	+
M fortuitum	—	—	—	—
M smegmatis	—	—	—	—

Specificity of antibody to infecting agent Table 4 gives the FAT results with diluted sera from the two series, using various mycobacteria as substrate In Series 1, the "early" sera were specific for the homologous type, while the 'late' sera were non specific With the "early sera, antibodies from both peaks 1 and 2 in the fracionation procedure were specific for the homologous type However peak 1 antibodies gave unwanted background staining and their reactions were difficult to read

In Series 2, the "early" sera reacted with *M bovis*, *M tuberculosis* and BCG and the "late" sera with other mycobacteria in addition

DISCUSSION

Use of the FAT in mycobacterial serology involves some technical difficulties Mycobacteria possess a strong autofluorescence which is bluish in colour in this optical system In order to distinguish it from the specific fluorescence, it is necessary to use a thin secondary filter which is as colourless as possible In some cases the best results are obtained with a pure ultraviolet excitation beam The problem of fixing the smears is still unsolved Previous experience had shown that both the heat and chemicals used reduced the fluorescence intensity It has subsequently been proved that heating at 65° for two hours, as used by *Jones et al* (1965), causes only a slight reduction in intensity and can therefore be recommended However, that temperature is rather close to the safety threshold for some mycobacterial species

The results obtained in this study show that it is possible to follow the course of an infection in rabbits by the level of circulating antibodies In contrast to the tuberculin reactions, the amount of circulating antibodies corresponds fairly well to the clinical state of the animals, as judged on the bases of the weight curves When comparing the results of the three groups in the two series, it might be visualized that the *multiplication of bacilli in vivo is more important for the reaction than* the number originally injected Whatever mechanism of antigen liberation is involved, the specificity of antibody for the infecting agent indicates that the reaction depends on the bacillus and not on some cellular destruction of the host organism

The serum fractionation results revealed some difference between the "early" and "late' sera It is a known fact that three main peaks will be observed in this system, and if present, immunoglobulin M will be found in the first peak and immunoglobulin G in the second The existence of a large first peak in the infected sera might be due to the high lipid content in such sera, since the lipids emerge with or before the macroglobulins However, the fact that the "early" sera had antibodies in peak one, in contrast to the "late" sera, suggests a shift between immunoglobulin M and immunoglobulin G as known from other immunization systems (*Cohen & Porter* 1964) The consequence of this hypothesis adds a new dimension to the evaluation of antibody dynamics However, the FAT is not entirely suitable for the demonstration of immunoglobulin M

The high specificity of the antibodies in Series 1 for the infecting bacilli suggests that the FAT can be used for typing mycobacteria According to the findings reported here, the best results would be obtained by bleeding the animals as early as possible during the infection and preferably by employing the immunoglobulin G fraction A sub-

sequent work will describe an example of use of the FAT in serotyping *M avium*

CONCLUSION AND SUMMARY

It was possible by means of the fluorescence antibody test (FAT) to follow the course of experimental infection in rabbits inoculated with *M avium* or *M bovis* The FAT results were in relation with the infection dose as regards slope of the titre curves and time of first response The antibodies demonstrated seemed to be indicative of infection and in their early phase, to be specific for the infecting agent As a consequence of these results, use of the FAT in mycobacterial serology is suggested, especially in the field of type identification Furthermore, its use in the diagnosis of infection should be considered

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INVESTIGATIONS ON THE ENZYMES AND TOXINS OF STAPHYLOCOCCI

Stimulation of Coagulase Biosynthesis by Glucose and Bicarbonate

By

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Received 5 v 66

During studies on the biosynthesis of staphylococcal α hemolysin attempts were made to replace the requirement of gaseous carbon dioxide by supplying glucose and potassium bicarbonate to the medium (13) Under these conditions the bacteria liberated acid from glucose which in turn reacted with the bicarbonate to produce carbon dioxide Interestingly enough a strain which did not produce coagulase in the presence of gaseous carbon dioxide was found to form coagulase when glucose and bicarbonate was supplied This phenomenon was studied further in order to provide a method for production of coagulase on a large scale in a form suitable to be incorporated into a vaccine

MATERIAL AND METHODS

Strain A laboratory strain of Staphylococcus aureus Walker , phage type 42 D (group IV) was stored in the lyophilized state It was grown on the same medium as was used for coagulase production

Media Two media were used for the cultivation of the bacteria nutrient broth and a casein hydrolysate medium They had the following composition

Nutrient broth contained 5 liters of beef extract (prepared by extracting at 95–100° C one kg of minced beef with 3 liters of distilled water) to which were added 100 g Bacto peptone 30 g NaCl and 20 g Na_2HPO_4 $12H_2O$ The mixture was boiled at pH 8 4 for five minutes filtered the pH adjusted to 7 4 and sterilized at 120° C for 30 minutes

Casein hydrolysate medium contained in ten liters sufficient acid hydrolysed casein (treated with charcoal acid pH) to give a final formol titrable nitrogen value of 0 15 mg per ml, 1 liter yeast dialysate representing 66 g dry yeast cells [illegible] the

of

the

rabbit plasma used for the determination of coagulase contained 0 02 per cent sodium merthiolate to prevent bacterial growth In the present study rabbit plasma was chosen as it was shown that staphylokinase either did not activate rabbit plasminogen (5) or gave only a low degree of activation (7) probably due to the content of antiplasmin in the plasma Even with human plasma it was demonstrated that precipitation of fibrin occurred within 24 hour whereas it took about 48 hours for staphylokinase to act and dissolve the [illegible] 6

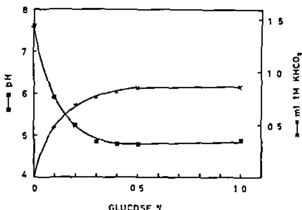


Fig 1

Relationship of initial glucose concentration to pH and the amount of bicarbonate needed to neutralize the acidity after 24 hours growth in nutrient broth

Test samples were diluted with 0 2 per cent peptone in 0 05M Tris HCl buffer of pH 7 6 which was sterilized by autoclaving at 120° C for 30 minutes The rabbit plasma containing 1 per cent sodium citrate was checked for absence of anti coagulase antibodies with the aid of a standard coagulase preparation before being used for the tests For this purpose serial two fold dilutions of the coagulase preparation was mixed with the test plasma and incubated at 37° C A similar series with the plasma in current use was run in parallel Test plasma which gave lower coagulase titres due to the presence of antibodies or inhibitors was rejected 0 1 ml of the test solutions was mixed with 0 4 ml of rabbit plasma and incubated at 37° C Periodic inspection for coagulation was made after 1 2 5 and 24 hours incubation One unit of coagulase was taken as the amount which just clotted 0 4 ml plasma after 24 hours incubation at 37° C. In general test samples containing 2 coagulase units clotted after 5 hours 3 units clotted after 2 hours and not less than 4 units clotted after 1 hour incubation Test solutions which clotted at the end of 1 hour were diluted and the dilutions tested for coagulase activity after incubation for 24 hours Preliminary ten fold dilutions and final intermediate dilutions were tested in order to ascertain the exact titre

pH and optical density The pH was measured using a Radiometer pH titrator with a combined glass electrode which was sterilized by treatment with ethanol The amount of 1M $KHCO_3$ required to neutralise the acid formed from glucose was determined using the same equipment provided with a magnetic valve The optical density of the cultures was measured at 600 mμ using a Bausch & Lomb Spectronic 20 colorimeter with the uninoculated medium as the blank set at zero Media containing Parker medium were acidified with a drop of 5N HCl to change the deep red colour to yellow before measuring the optical density

EXPERIMENTAL

Effect of Glucose on Acid Production

Nutrient broth containing different amounts of glucose was inoculated with 0 1 ml of a 18 hour culture of the Staph aureus strain After 24 hours the pH and the amount of bicarbonate needed to neutralize the acid formed from glucose was determined The results shown in Fig 1 indicate the effect of various amounts of glucose A rapid fall in pH from 7 60 to 4 85 occurred when the amount of glucose was increased from 0 to 0 3 per cent With further increase up to 1 0 per cent

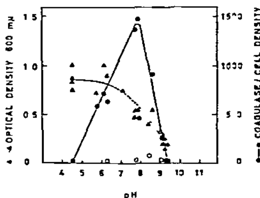


Fig 2

Relationship of pH to growth and coagulase production in nutrient broth
Open triangles and circles denote respectively growth and coagulase obtained in the absence of either glucose or bicarbonate or both

the pH remained stationary around 4 80 The amount of $KHCO_3$ needed to neutralise the acid formed from glucose increased gradually up to 0 5 per cent glucose concentration

Production of Coagulase in Nutrient Broth

The effect of different concentrations of glucose and bicarbonate on the growth, acid production and coagulase biosynthesis is represented in Table 1 In the presence of 0 5 per cent or more of glucose the pH decreased from 7 8 to 4 5 after 24 hours growth The presence of increasing amounts of bicarbonate prevented the decrease in pH by interacting with the acid formed from glucose and liberating carbon dioxide Bubbles of gas were seen in cultures containing lower amounts of both supplements whereas the cultures containing higher concentrations were foaming With 2 per cent bicarbonate giving an initial pH of 9.20 the pH did not decrease appreciably even with higher amounts of glucose and the growth as judged by optical density of the cultures at 600 mμ, was also diminished Coagulase activity in the supernatants was not demonstrable when the final pH was 4.55 or above 9 20 Optimal coagulase titres were obtained when the concentrations of both glucose and bicarbonate were 0.5 or 1 0 per cent

The relationship of growth to final pH of the culture and of coagulase production to growth at various pH values are represented in Fig 2 After 24 hours incubation maximum growth was found to occur in media having pH 6 5 and lower pH values, but diminished considerably as the pH rose to 9 0 The coagulase activity per unit optical density was negligible above pH 9 0 but increased rapidly with decrease in pH to a maximum at 7 90 Further decrease in pH reduced the formation of coagulase and at pH 4 5 little activity was demonstrable in the culture Thus cultures with a final a pH of 7 90 produced maximal amounts of

TABLE 1

Effect of Different Amounts of Glucose and Bicarbonate on pH Growth and Coagulase Biosynthesis in Nutrient Broth by Staph aureus after 24 Hours Incubation at 37° C

Glucose %	pH KHCO$_3$ %					Optical density at 600 mμ KHCO$_3$ %					Coagulase units per ml KHCO$_3$ %				
	0	0 1	0 5	1 0	2 0	0	0 1	0 5	1 0	2 0	0	0 1	0 5	1 0	2 0
0	7 80	8 45	9 10	9 30	9 40	0 475	0 400	0 325	0 250	0 200	10	30	10	0	0
0 1	6 30	7 15	8 65	9 10	9 30	0 900	0 750	0 550	0 300	0 160	10	100	500	100	0
0 5	4 55	4 55	6 38	7 90	9 15	0 750	0 845	0 790	0 510	0 190	0	0	500	750	0
1 0	4 55	4 55	6 15	7 80	9 15	0 825	0 850	1 050	0 550	0 225	0	0	750	750	0
2 0	4 55	4 55	5 80	7 90	9 20	0 840	1 000	0 725	0 550	0 310	0	0	500	250	0
uninoculated control	7 88	8 35	8 95	9 13	9 20										

coagulase although growth was suboptimal In the absence of either glucose or bicarbonate coagulase production (open circles) was negligible though growth (open triangles) was not as much affected

Production of Coagulase in a Casein Hydrolysate Medium

The effect of different amounts of glucose in the presence of 1 0 per cent bicarbonate on growth and coagulase formation on a caseinhydrolysate medium was studied Coagulase formation was negligible on this medium in the absence of either glucose or bicarbonate or both (Table 2) Growth was diminished in the presence of bicarbonate alone and increased as the glucose concentration was raised In parallel coagulase formation also increased giving 320 units per ml up to 0 75 per cent glucose, but further increase of glucose tended to diminish the content to coagulase

TABLE 2

Coagulase Production and Growth in Casein Hydrolysate Medium in the Presence of Glucose and Bicarbonate

Glucose %	KHCO$_3$ %	pH Initial	pH Final	Opt density 600 mμ	Coagulase units per ml	Coagulase units per unit optical density
0	0	7 60	7 40	2 00	10	5
0	1	8 35	8 40	0 30	0	0
0 025	1	8 35	8 10	0 60	10	13
0 050	1	8.35	8 10	0 90	10	9
0 100	1	8 35	7 85	0 90	160	177
0 250	1	8.35	7.20	1 39	320	230
0.500	1	8.35	6 62	1 15	320	276
0 750	1	8.35	6 50	2 00	320	160
1 000	1	8.35	6 60	2.20	240	108
1 000	0	7 60	5 25	1 15	10	8

Production of Coagulase in Casein Hydrolysate Medium Supplemented with Parker Medium

The amount of coagulase produced on the casein hydrolysate medium was low when compared with that obtained on nutrient broth In order to supply any additional factors that may be needed Parker medium (11) was added as a supplement in 10 per cent concentration The complete Parker medium contains antibiotic supplements which was omitted In the presence of 1 per cent glucose, the effect of different concentrations of bicarbonate was studied Bicarbonate concentrations between 0 375 and 2 0 per cent yielded supernatants having 1000 coagulase units per ml (Table 3) and 1227 coagulase units per unit optical density at pH 7 30 Thus, the addition of Parker medium stimulated the production of coagulase to the same extent as that formed in nutrient broth

TABLE 3

Effect of Glucose and Bicarbonate on Growth and Coagulase Production by Staph aureus Walker on Casein Hydrolysate Medium Containing 10 per cent Parker Medium

Glucose %	KHCO$_3$ %	pH Initial	pH Final	Opt density 600 mμ	Coagulase units per ml	Coagulase units per unit optical density
1	0	7 05	4 60	0 950	0	0
1	0 125	7 58	4 65	1 150	100	87
1	0 250	7 90	4 70	1 140	500	439
1	0 375	8 10	4 90	1 625	1000	615
1	0 500	8 20	5 25	1 560	1000	641
1	0 625	8 25	5 40	1 625	1000	615
1	0 750	8 35	5 85	1 750	1000	571
1	1 000	8 45	6 35	1 925	1000	519
1	1 500	8 60	6 80	1 410	1000	708
1	2 000	8 70	7 30	0 815	1000	1227
1	2 500	8 78	8 00	0 715	500	699

Purification of Coagulase by Gelfiltration on Sephadex G-100

For the preparation of coagulase, the bacteria were grown for 24 hours in 1 liter bottles containing 500 ml of nutrient broth with a final concentration of 1 per cent each of glucose and potassium bicarbonate The cultures were centrifuged at 3000 g for 1 hour and the supernatant after addition of 0 01 per cent sodium merthiolate, was concentrated to a tenth of its volume by ultrafiltration at 5° C using Berkefeld filters

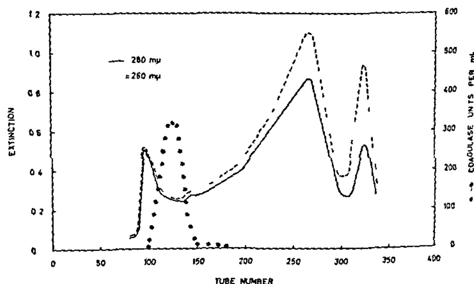


Fig 3

Staphylococcal coagulase Fractionation on Sephadex G 100

coated with 10 per cent collodion The concentrate was centrifuged at 30000 g for 20 minutes at 4° C to sediment any remaining cells 30 ml of the clear supernatant was chromatographed on Sephadex G 100 gel (Pharmacia, Uppsala) using a column size of 5 cm diameter and 150 cm length and employing 0 05M Tris HCl buffer of pH 7 6 with 1 per cent n butanol Effluent samples were collected in 10 ml lots on an automatic fraction collector Extinction measurements at 260 and 280 mμ were made using a Beckman spectrophotometer model DU using 1 cm light path cuvettes Three peaks were found all of which had a higher 260 than 280 mμ absorption (Fig 3) Coagulase activity was found to occur in the fractions coming after the first peak with maximal activity in tube 125 The position of the coagulase fraction indicated a molecular weight around 90000

DISCUSSION

The possession of coagulase by a staphylococcal strain is generably considered to indicate that it may be pathogenic Much work has been done by *Tages & Drummond* (12) in the preparation and purification of coagulase but little is known concerning the precursors for its biosynthesis as well as its mode of action The importance of obtaining coagulase in a relatively pure form in order to evaluate its protective function need not be emphasized For the preparation of coagulase in large quantities conditions optimal for its biosynthesis had to be worked out first

It seemed therefore to be of great interest to study the stimulatory effect of glucose and bicarbonate on the formation of coagulase A survey of the literature revealed that coagulase had always been produced on media containing glucose The addition of serum (1), serum albumin (3) and peptones (9) greatly enhanced coagulase formation It was shown by *Rogers* (10) and by *Duthie & Haughton* (4) that the addition of sodium β glycerophosphate to a casein hydrolysate medium promoted coagulase biosynthesis These results together with the present findings of the effect of bicarbonate suggest that the stimulatory activity of these supplements were due to their buffering action However, the effect of substances other than bicarbonate to stabilize pH must also be studied Similar studies are also to be made with regard to the Parker medium which activated coagulase formation It was shown earlier that glutamate, which is a component of Parker medium, can stimulate coagulase biosynthesis (8) During purification by gel filtration chromatography on Sephadex G 100 it was found that coagulase occurred next to the void volume thus indicating a molecular weight of 90 000 The two preparations of purified coagulase reported in the literature gave molecular weight values of 44 000 (4) and between 5000 and 10 000 (9) The preparation reported in this study was not subjected to either salt or acid precipitation unlike those reported in

the literature It is possible that such precipitation treatments may break the larger molecule into smaller fragments which are still enzymatically active, as contended by *Tager & Drummond* (12) It is also possible that the coagulase produced by different strains have different molecular size particularly as four different antigenically distinct forms were reported (2)

SUMMARY

The production of coagulase in the presence of varying amounts of glucose and potassium bicarbonate in nutrient broth and casein hydrolysate medium has been studied Under optimal concentrations of these supplements and a final pH of between 7 3 and 7 9 maximal amounts of coagulase were produced on both media, whereas growth was suboptimal These observations provide a basis for the production of large amounts of coagulase for preparation of a staphylococcal vaccine

Gelfiltration on Sephadex G 100 indicated that coagulase has a molecular weight around 90 000

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REACTION OF ANTINUCLEAR FACTORS WITH POLYMORPHONUCLEAR LEUCOCYTES

1 *Absorption Studies*

By

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Received 7 x 66

Antinuclear factors (ANF) as determined by the immuno fluorescent antibody technique are generally considered to be organ and species non specific, reacting with all mammalian cell nuclei The existence of ANF reacting specifically with the nuclei of granulocytes and not with other mesenchymal or epithelial cells has however been demonstrated (4 10, 17) and leucocyte specific ANF have now been found as the only ANF present in sera from patients with rheumatoid arthritis Felty's syndrome S L E and a few other diseases (6)

Determining the incidence of this factor in human sera difficulties arose from the investigation of sera containing ANF reacting also with other nuclei since it was uncertain whether the reaction with granulocytes was due exclusively to the leucocyte specific ANF or to other ANF cross reacting with the granulocytes As the question of the existence of organ specific ANF is important when the etiology and pathogenesis of S L E is discussed the object of the present study has been to reveal the presence of leucocyte specific ANF in sera containing ANF reactive also with other cell nuclei In order to provide a simple method to determine the incidence of leucocyte-specific ANF in human sera an attempt has been made to find out whether the ANF activity with granulocytes exclusively is caused by one factor the leucocyte-specific ANF or whether sera containing other ANF reactive with other nuclei cross react with nuclear substances in the polymorphonuclear granulocytes

METHODS

The indirect fluorescence antibody technique was performed as described in detail in a previous report (5) with the following modifications

As *nuclear substrate* blood smears and thyroid sections were used The smears were prepared immediately after the blood had been drawn and allowed to dry for 5 minutes In all cases serum was applied to the slides within 15 minutes since this procedure has been shown to give reproduc ble results with 19 sera containing only leucocyte specific ANF (3) No fixation was used but advantage was taken

of the adherence of leucocytes to glass prewarmed to 37° C The thyroid gland was obtained at operation from a patient with toxic goitre

The *antisera* used for conjugation with fluorochrome were prepared in rabbits against whole human gamma globulin and against human Ig G Ig A and Ig M immunoglobulins respectively Only selected antisera against human gamma globulins which show only precipitating lines against the immunoglobulins in immuno electrophoresis were used for conjugation The three specific antisera against human Ig G Ig A and Ig M globulins were obtained from Centraal Lab., Roode Kruis Amsterdam and were shown immunoelectrophoretically to give only one line at the expected place using whole human serum as antigen and were furthermore shown to react only with homologous myeloma proteins The antisera were conjugated with 0 025 mg fluorescein isothiocyanate per mg protein giving a final fluorochrome protein ratio of 7 3 $\times 10^{-3}$ of the conjugate against human gammaglobulin and a ratio of 4 1 $\times 10^{-3}$ of the three specific antisera (The investigation of the immuno chemical specificity of the antisera was kindly performed by B Mansa Biophysical Department Statens Seruminstitut) The conjugates were absorbed with acetone dried guinea pig liver powder immediately before use

The *sera* were frequently stored for several months before use and applied undiluted to the slides at room temperature

The *microscope* was a Leitz Ortholux (Zernicke) with equipment for combined light field fluorescence and phase contrast microscopy The ultraviolet light source was a high pressure mercury lamp (Osram HBO 200) An UG1 exciter filter was used together with an UV absorbing filter

Preparing of Nuclei for Absorption

A Human *leucocytes* were obtained from a healthy donor blood group O Rh negative The blood was heparinized or defibrinated by vigorous shaking for 10 minutes After sedimentation in a 3 per cent solution of dextran in saline in a 2 1 dextran blood ratio in 5 ml tubes the supernatant was removed and centrifuged for 10 minutes at 1000 g The sediment containing 75 per cent of the leucocytes and contaminated with 1 per cent erythrocytes was washed three times with buffered saline Isolated nuclei were prepared by treating the sediment from the final wash for half an hour with 0 2 per cent citric acid which was then removed by washing the sediment with buffered saline After the final wash the nuclei were resuspended in buffered saline adjusted to about 10^6 nuclei per ml and used immediately The suspension prepared in this manner was a mixture of nuclei of lymphocytes and granulocytes (ratio 1 3) Almost all the granulocytes had lost the cytoplasm while several lymphocytes had a small rim of cytoplasm attached

B Human *lymphocytes* were prepared from two different sources B 1) Blood from the same donor as in A was defibrinated and brought to sediment in a fresh solution of 3 per cent gelatin in saline at 37° C for half an hour The top of the supernatant was removed washed three times with buffered saline and treated with citric acid as described above After the final wash with saline the amount of cell nuclei was adjusted to 10^3 nuclei per ml saline This procedure yielded a lymphocyte preparation contaminated with approximately 10 per cent granulocytes B 2) Blood was collected from a patient with chronic lymphatic leukaemia with a leucocyte count of 24000 per cmm of which 90 per cent were lymphocytes After defibrination the blood was allowed to pass through a column packed with glass wool and glass beads This procedure yielded a suspension of lymphocytes contaminated with about 1 per cent granulocytes The isolated nuclei were prepared as described above The amount of nuclei was adjusted to 10^6 nuclei per ml saline

C *Thyroid nuclei* were obtained from a human thyroid gland removed on account of a toxic goitre To isolate the nuclei the method described by *Hogeboom* (12) was used 50 g of the gland was minced immediately after the removal and in a solution of 0 25 M sucrose at 4° C disrupted in a high speed kitchen robot for 30 seconds homogenized in a glass tissue grinder and filtered through gauze The nuclei were separated by a differential centrifugation in 0 34 M sucrose at 1000 g for 15 minutes The sediment was washed three times and resuspended in saline By phase contrast microscopy and staining with Giemsa solution the isolated thyroid nuclei were found to be contaminated with a few white blood cells

Testing of Sera and Absorption Procedure

Before and after absorption all sera were teste l by means of the four d fferent conjugated antisera described above in order to establish the immunoglobulin class of the ANF and the amount of ANF reacting with granulocytes lymphocytes and thyroid nuclei The reaction was est mated as weakly positive (+) med um positive (++) and strongly positive (+++) from the appearance of the intensity of the flourescence

In pilot essays the quantity of nuclei necessary to absorb a serum containing ANF in a titre of 1/400 was evaluated and in the absorption procedures the same quant ties were used 0 2 ml of each serum was incubated with the sed ment of 0 3 ml of the suspensions of A B 1 B 2 and C for 1 hour at 37° C under occasional stirring The absorpt on was performed in small plastic tubes wh ch were then centrifuged for 15 minutes at 10 000 g

If no fluorescent nuclei were found in the blood smears the slides were always observed by phase contrast microscopy in order to make sure that granulocytes and lymphocytes actually were present in the smears

MATERIAL AND RESULT

Thirty eight serum samples were investigated The sera were collected from patients with S L E rheumatoid arthritis and Feltys syndrome The diagnostic criteria used for selection have been given in a previous report (5) The present study was restricted to sera giving a high titered homogeneous fluorescence of all nuclei or containing only a leucocyte specific ANF 15 sera were absorbed with the various mixtures of granulocytes and lymphocytes while 28 sera were absorbed with isolated thyroid nuclei

From Table 1 it is seen that 5 of 15 sera contained as the only ANF demonstrable an ANF reacting with granulocytes while 10 sera con tained ANF reacting with thyroid nuclei and lymphocytes also In 5 of these 10 sera a more intense fluorescence of the granulocytes than that of other nuclei was noticed as a constant feature Table 1 further shows the ANF activity given by these sera after *absorption with A* (a mixture of granulocytes and lymphocytes ratio 3 1) *with B 1* (isolated lymphocytes from a normal donor contaminated with a small amount of granulocytes ratio 9 1) and *with B 2* (isolated nuclei of lymphocytes from a patient with chronic lymphatic leukaemia) The reaction of ANF with the nuclei of the white blood cells in blood smears and with thyroid nuclei was demonstrated with the conjugated anti serum against human gamma globulins before and after absorption

Absorption with A removed all ANF activity with granulocytes and lymphocytes while the ANF reacting with thyroid nuclei seemed un changed except for a small fall in the fluorescent intensity in three cases (sera Nos 6 13 15)

Absorption with B 1 abolished ANF activity with the lymphocytes In five cases the ANF reaction with granulocytes became negative probably owing to contamination of the absorbent with granulocytes The ANF reacting with thyroid nuclei was not abolished by this ab sorption procedure except for a small fall in fluorescent intensity in serum No 8

TABLE

The Nuclear Reactivity of 15 Sera before and after Absorption with A) a Mixture of Lymphocytes with a small Contamination of Granulocytes (ratio 9 1) and B 2) Isolated ANF a Conjugated Antiserum against Human

Serum No	Before absorption			After		
	Sera reactive with nuclei of			Nuclear reactivity A Granulocytes & lymphocytes		
	Granulocytes	Lymphocytes	Thyr nuclei	Granulocytes	Lymphocytes	Thyr nuclei
1	+++	0	0	0	0	0
2	+++	0	0	0	0	0
3	+++	0	0	0	0	0
4	+++	0	0	0	0	0
5	+++ (1/256)	0	0	0	0	0
6	+++ (1/64)	++	++ (1/16)	0	0	+
7	+++	++	++	0	0	++
8	+++ (1/64)	++	++ (1/16)	0	0	++
9	+++ (1/64)	+	+ (1/2)	0	0	+
10	+++	++	++	0	0	++
11	+++	+++	+++	0	0	+++
12	+++	+++	+++	0	0	+++
13	+++	+++	+++	0	0	++
14	+++	+++	+++	0	0	+++
15	+++ (1/256)	+++	+++ (1/256)	0	0	++

+++ = *strong and bright fluorescence of all nuclei*

Absorption with B 2 removed all ANF activity with lymphocytes The ANF reacting with granulocytes and thyroid nuclei were still present and seemed unchanged except in 5 sera in which a slight fall in the fluorescent intensity was seen

Although the absorptions indicate that isolated granulocytes and lymphocytes are capable of absorbing specifically the ANF reacting with these nuclei in blood smears but incapable of removing the ANF which react with other nuclei (besides thyroid nuclei these ANF have been found to react with nuclei of rat liver and guenon monkey adrenal tissues) a fall in titre was noticed in some cases This loss of activity was however found unrelated to the titre (e g the high titered serum No 15 was partially absorbed while serum No 9 with a low titered ANF reaction with thyroid nuclei was unaffected) and an attempt was made to see whether a specific removal of an ANF of one of the three immunoglobulin classes had occurred since it has been shown that

I

Isolated Nuclei of Granulocytes and Lymphocytes (ratio 3 1), b 1) Isolated nuclei of Nuclei of Lymphocytes from a Patient with Lymphatic Leukaemia For Determination of Gamma Globulins has been Used

absorption						
of sera after absorption with						
B 1 Lymphocytes & granulocytes			B 2 Lymphocytes			Serum No
Granulocytes	Lymphocytes	Thyr nuclei	Granulocytes	Lymphocytes	Thyr nuclei	
0	0	0	+++	0	0	1
0	0	0	+++	0	0	2
++	0	0	+++	0	0	3
+++	0	0	+++	0	0	4
+++	0	0	+++	0	0	5
+++	0	++	+++	0	+	6
+++	0	++	+++	0	+	7
0	0	+	++	0	++	8
0	0	+	+	0	+	9
0	0	++	++	0	++	10
++	0	+++	++	0	++	11
+++	0	+++	+++	0	+++	12
+++	0	+++	+++	0	++	13
+++	0	+++	+++	0	+++	14
+++	0	+++	++	0	+	15

+ to ++ = weak to medium fluorescence of nuclei

ANF are heterogeneous antibodies which have been found in the Ig G, Ig-A, and Ig M immuno-globulin classes of human sera (1)

An attempt was therefore made to find out whether all ANF belonging to the three human immuno globulin classes Ig-G, Ig-A and Ig-M, found in sera before the absorption, were still present after the absorption Table 2 shows the result of testing the sera before and after absorption with the various isolated nuclei (A, B 1 and B 2) with the use of conjugated specific antisera against the human Ig-G, Ig-A, and Ig-M immuno-globulins

Before absorption all 15 sera contained Ig G ANF reacting with granulocytes and in 10 sera also with lymphocytes and thyroid nuclei, except in two cases (sera Nos 10 and 12) where no Ig-G ANF was found to react with lymphocytes None of the five leucocyte-specific ANF reacted with the Ig-M or Ig A conjugate, while seven Ig-M ANF were found in the mixed sera reacting with granulocytes, five with

TABLF

The Reactivity of ANF before and after Absorption with Various Mixtures of Isolated γA have

Serum No	Before absorption			After		
	Sera reactive with nuclei of			Nuclear reactivity A Granulocytes & lymphocytes		
	Granulocytes	Lymphocytes	Thyr nuclei	Granulocytes	Lymphocytes	Thyr nuclei
1	γG	0	0	0	0	0
2	γG	0	0	0	0	0
3	γG	0	0	0	0	0
4	γG	0	0	0	0	0
5	γG	0	0	0	0	0
6	γGM	γGM	γGM	0	0	γM
7	γGM	γG	γGM	0	0	γM
8	γGM	γG	γGM	0	0	γM
9	γG	γGM	γGM	0	0	γM
10	γGM	γM	γGM	0	0	γM
11	γG	γG	γGM	0	0	γM
12	γGM	γM	γGM	0	0	γM
13	γGM	γGM	γGM	0	0	γM
14	γG	γG	γGA	0	0	γA
15	γGM	γG	γGM	0	0	γM

γG γA and γM Serum is shown to have ANF of the γG γA or γM immunoglobulin classes

lymphocytes and nine with thyroid nuclei In only one case was an Ig-A ANF found, and this factor reacted only with the thyroid nuclei

After absorption with A it was evident that all Ig-G ANF activity with granulocytes and lymphocytes and even with thyroid nuclei was abolished The Ig-M ANF, which was left reacting with thyroid nuclei, proved impossible to remove by further absorption with isolated nuclei of granulocytes and lymphocytes

After absorption with B 1 it was found that, except for one case (serum No 7), no Ig G ANF reacting with thyroid nuclei was absorbed Both the Ig-G and Ig-M ANF reacting with granulocytes were removed in a few sera The absorption yielded two sera which contained Ig G ANF reacting selectively with thyroid nuclei (Nos 8 and 9) and one serum with Ig G and Ig-M ANF reacting only with thyroid nuclei (No 10)

The absorption with B 2 abolished the reaction with lymphocytes in all 10 sera, while all Ig G and Ig-M and Ig A ANF reacting with granulocytes and thyroid nuclei found in sera before absorption were still present after the absorption

The results of the absorptions indicate that nearly all ANF reactivity

uclei For Determination of ANF Conjugated Antisera against Human γG γM, and een Used

bsorption						
f sera after absorption with						Serum No
B 1 Lymphocytes & granulocytes			B 2 Lymphocytes			
Granulocytes	Lymphocytes	Thyr nuclei	Granulocytes	Lymphocytes	Thyr nuclei	
0	0	0	γG	0	0	1
0	0	0	γG	0	0	2
γG	0	0	γG	0	0	3
γG	0	0	γG	0	0	4
γG	0	0	γG	0	0	5
γGM	0	γGM	γG	0	γGM	6
γGM	0	γM	γGM	0	γGM	7
0	0	γG	γGM	0	γGM	8
0	0	γG	γG	0	γGM	9
0	0	γGM	γGM	0	γGM	10
γG	0	γGM	γG	0	γGM	11
γG	0	γGM	γGM	0	γGM	12
γGM	0	γGM	γGM	0	γGM	13
γG	0	γGA	γG	0	γGA	14
γGM	0	γGM	γGM	0	γGM	15

of the Ig-G immunoglobulin class can be absorbed with granulocytes, while the ANF of high molecular weight (Ig-M ANF) is unaffected by this treatment The absorption has, however, in 3 of 10 sera revealed the presence of an antibody of low molecular weight (Ig-G ANF) with nuclear reactivity against thyroid nuclei, but not against granulocytes or lymphocytes The results further point to the existence of a lymphocyte-specific ANF, since the reactivity of ANF with lymphocytes was selectively abolished by absorption of the sera with isolated nuclei of lymphocytes

No evidence of the existence in the 10 "mixed" sera of ANF that react specifically with the granulocytes was obtained Therefore, an attempt was made to reveal this factor by absorbing the sera with isolated nuclei of a human thyroid gland Ten sera containing only leucocyte-specific ANF and 18 sera from patients with S L E , containing high titered ANF and reacting with granulocytes and thyroid nuclei as well, were selected for absorption

The result of the absorption of these 28 sera with C (isolated thyroid nuclei) are seen in Table 3 Four of the 10 sera containing only leucocyte specific ANF were rendered negative while the fluorescent intensity given by these sera with granulocytes was unchanged in 6 cases

After the absorption, 15 of the 18 sera still reacted with an intense fluorescence with the nuclei of granulocytes in blood smears, while 7 sera still reacted with thyroid nuclei Of these 7 sera, 2 showed a decreased titre of the ANF reactive with thyroid nuclei and a further 2 sera showed only a speckled type of ANF with thyroid nuclei In 8 of 18 sera from patients with S L E, the absorption thus revealed the presence of ANF reacting exclusively with granulocytes in blood smears In 3 cases all ANF reactivity was abolished by this treatment

TABLE 3

ANF Reactivity of 28 Sera Tested before and after Absorption with Isolated Thyroid Nuclei in an Amount Sufficient to Absorb Serum Containing ANF in a Titre of 1/400

Before absorption: Number of sera reactive with nuclei of Granulocytes	Before absorption: Thyroid nuclei	After absorption: Number of sera reactive with nuclei of Granulocytes	After absorption: Thyroid nuclei
10/10	0/10	6/10	0/10
18/18	18/18	15/18	7/18*

* Two sera showed a fall in titre

DISCUSSION

The granulocyte-specific ANF, which have previously been demonstrated in sera from patients with rheumatoid arthritis and Felty's syndrome, have been found in only few sera from patients with S L E as the only ANF detectable by the fluorescent antibody technique (6) The absorption experiments here reported have revealed with a balanced absorption—using an amount of nuclei sufficient to absorb a serum containing ANF reacting with human thyroid nuclei in a titre of 1/400—a granulocyte-specific ANF in 8 of 18 lupus sera which before the absorption apparently only possessed organ-non specific ANF reacting with all kinds of nuclei This is, however, probably a minimum estimate of the real incidence, since it was found that the suspensions of thyroid nuclei used for absorption also absorbed to a certain extent the leucocyte-specific ANF in sera containing only this factor The apparently "non-specific" absorption of the granulocyte-specific ANF with thyroid nuclei may possibly be due to a contamination of the thyroid nuclei with white blood cells, since such a contamination was observed directly

The result of the absorption of sera with isolated nuclei of lymphocytes contaminated with some granulocytes (B 1) is difficult to interpret with respect to the existence of organ specific ANF, but might indicate the presence of a lymphocyte specific ANF Absorption of sera

with an apparently pure suspension of isolated nuclei of lymphocytes (B 2) results in a loss of both Ig G and Ig M ANF reacting with lymphocytes in blood smears, but not of ANF reacting with other nuclei; it also points to the existence in these sera of lymphocyte specific ANF In 4 cases a fall in the intensity of the fluorescence of ANF reacting with other cell nuclei was noticed (sera Nos 6, 9, 11, 14), which indicates a cross-reactivity of this factor with other cell nuclei It might, however, also be explained by the fact that the suspensions of lymphocytes nevertheless have been contaminated with some granulocytes, since it is almost impossible to prepare absolutely pure suspensions of one sort of nuclei

ANF which react with thyroid nuclei, but not with granulocytes, have been revealed in some sera Low titered ANF (e g serum No 9, Table 1), which react with thyroid nuclei proved impossible to remove with isolated granulocytes, while some high titered ANF (e g serum No 15, Table 1) showed a great fall in fluorescent intensity The reason for these findings seems to be a "specific" absorption of only ANF of low molecular weight (Ig G ANF), while the ANF of high molecular weight (Ig-M), which react with thyroid nuclei and other nuclei but not with granulocytes, were unaffected by this absorption Although it seems unlikely that this finding could be due only to differences in the amount of Ig G and Ig M ANF in the sera, it would have been advisable to have estimated the actual titres of Ig G, Ig-A, and Ig M ANF and not only the intensity of the fluorescence in sera before and after absorption Furthermore, it has to be considered whether the non-reactivity of some of these factors could be caused by an inaccessibility of the nuclei to the antibody due to the molecular size of the antibody (13) or to differences in the biophysical state of the nuclei used for testing for ANF It has previously (1), and also in the present study, been shown that high molecular weight ANF react with granulocytes in blood smears, and even leucocyte specific ANF—when found as the only ANF demonstrable—have in a few cases been found to belong to the Ig-M immunoglobulins (6) It is more likely that differences in the biophysical state of the nuclei may account for the non-reactivity of these factors with antigens in blood smears, since the nuclei have been treated in a different way While the nuclei of the thyroid sections have been frozen to —70° C, cut in a cryostat and then thawed, the granulocytes and lymphocytes in blood smears were unfixed, used within few minutes and the nuclei still surrounded by the cytoplasm through which the antibody has to penetrate

The results of the absorption experiments performed point conclusively only to the existence of one organ specific ANF within the limits of the organs tested a granulocyte-specific ANF Some evidence of the existence of a lymphocyte-specific ANF and an ANF that reacts with thyroid and other nuclei but not with granulocytes has been ob-

tained but these findings may be due entirely to the technical circumstances outlined above

A few earlier reports suggest that ANF might exhibit limited nuclear reactivity (2, 9, 10, 11, 18), but it has conclusively been demonstrated that ANF exists which reacts exclusively with granulocytes in blood smears (4, 17), and the clinical and serological data of some patients with granulocyte specific ANF have recently been published (6) ANF which react with lymphocytes, but not with granulocytes, have been found by *Gocken* (10) in a serum from one patients only, but similar factors seem to be present in sera from patients with hepatic cirrhosis (7) Recently, *Fellkamp* (8) using 6 different nuclear antigens for the determination of ANF in 750 sera, demonstrated the presence of 6 sera reacting only with leucocytes in blood smears, 9 sera reacting selectively with thyroid nuclei, 12 sera reacting exclusively with gastric mucosa nuclei and 17 sera reacting only with nuclei of adrenal cortical tissues By the same technique similar, apparently organ-specific, ANF have been found in sera from patients with hepatic cirrhosis (7) Whether the difference in nuclear reactivity solely reflects variation in the sensitivity of technique or is caused by the existence of several different antinuclear globulins is, however, unknown

Experimentally, no organ-specific ANF have been demonstrated By the fluorescent antibody technique it has been shown that ANF—although of low titre—which can be demonstrated in sera from rabbits immunized with isolated calf thymus nucleoprotein, cross-react with various cell nuclei (15) Similar antisera against isolated nuclei from different organs all show the same L E -cell-like lesions when incubated with granulocytes (14) Some objections have been raised against the conclusions of these experiments, since it was not conclusively demonstrated that the L E -cell like lesions produced by these antisera and the L E cell phenomenon given by lupus sera were identical (16), and since the suspensions of nuclei used for immunization had probably been contaminated with some white blood cells of which the granulocytes have several strong antigens

The present report has shown that although sera from patients with S L E and rheumatoid arthritis contain apparently organ non specific ANF reacting with all kinds of nuclei, these sera may also contain ANF with very selective nuclear reactivities the total number of which cannot be stated without further analysis It seems, however, important in investigations of the correlation of the occurrence of ANF to clinical and biochemical findings, as well as in determining the cytotoxic effect of these antibodies, to consider the limited nuclear reactivity that some of these antinuclear globulins possess In this connection, it is interesting to notice that several patients with granulocyte-specific ANF in sera show granulocytopenia

SUMMARY

Ten sera containing granulocyte specific ANF as the only ANF detectable by the immuno fluorescent antibody technique and 28 sera from patients with S L E and rheumatoid arthritis which contained apparently organ non specific ANF reacting with nuclei of several organs were absorbed with various mixtures of isolated nuclei Before and after absorption the nuclear reactivity of these sera was tested by the fluorescent antibody technique employing specific antisera against human Ig G Ig A and Ig M immunoglobulins

Absorption of 18 S L E sera containing organ non specific ANF with isolated thyroid nuclei revealed the existence in half of these sera of high titered ANF which—within the limits of the organs tested—exclusively reacted with granulocytes in blood smears This is however probably a minimum estimate of the real incidence of this organ specific ANF in S L E sera since the absorbent to a certain extent also absorbed the granulocyte-specific ANF

The absorption of sera with a pure suspension of isolated nuclei of lymphocytes resulted in a loss of both Ig G and Ig M ANF activity with lymphocytes in blood smears while ANF of different immuno globulin classes reacting with other nuclei were left unaffected in sera The results thus indicate the existence of a lymphocyte specific ANF in some sera from patients with S L E and rheumatoid arthritis

Absorption of sera with mixtures consisting largely of isolated nuclei of granulocytes abolished all Ig G ANF activity in sera while ANF of high molecular weight (Ig M ANF) which react with thyroid and other nuclei from solid organs but not with granulocytes were left unaffected in sera

The absorption experiments performed thus point to the existence of three different antinuclear factors in the sera investigated a granulocyte specific ANF a lymphocyte specific ANF and an ANF which reacts with thyroid and other nuclei but not with granulocytes in blood smears

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EFFECT OF INHIBITORS AND RESPIRATORY FACTORS ON THE GROWTH OF RESPIRATORY SYNCYTIAL (RS) VIRUS IN HEP 2 CELL CULTURES

By

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Received 1 vi 66

The importance of the Respiratory syncytial (RS) virus as an aetiological agent in respiratory disease in infancy and childhood is well established The ecology of infection and the biological properties of the virus are now well known (8), although it is only during the past 6 7 years that the virus has attracted interest on a worldwide scale A potent RS virus vaccine would be of great importance in infancy and early childhood Unfortunately apart from the fact that it is very labile, this agent usually grows to only moderately high titres in tissue culture Adaptation of this virus to suckling mice has also been achieved, but so far only low titres have been reached in mice (7) The difficulties in the manufacture of an effective vaccine are increased by these growth characteristics

Comparatively little is known about the factors which promote or inhibit the growth of this virus in tissue culture The development of syncytia is influenced by the composition of the maintenance medium (14) and the type of tissue culture chosen for growth (4, 21) The growth in Hep 2 cell cultures can be accelerated by incorporating proflavine in the maintenance medium (13)

The Hep-2 cell line has been developed from human tissue originally derived from a case of cancer of the larynx It has been found (16) that Hep 2 cells have a glucose consumption and an acid production which is similar to HeLa cells but lower than normal human embryonic skin and kidney cells

The growth of RS virus in Hep-2 cell cultures has been followed after addition to the maintenance medium of compounds whose influence on glycolysis the citric acid cycle or oxidative phosphorylation is established The experiments to be described suggest that the multiplication of RS virus in Hep-2 cells is less dependent on oxidative processes involving the tricarboxylic acid cycle than on other pathways of energy yielding reactions

MATERIAL AND METHODS

Tissue culture Only Hep 2 cells were employed They were maintained in passage in Roux bottles and grown in 150 × 12 mm tubes and in 5 cm plastic Petri dishes as described previously (13)

Preparation of virus stock The American Long strain was grown in Roux bottles and was harvested and stored as described (13)

Virus titration This was done in tubes as earlier described (13) with the following modification five fold dilutions of the sample to be assayed and three tubes per dilution were employed

Inhibitors respiratory factors and other compounds used in the experiments to be described 1) Sodium iodoacetate (Fluka A G), 2) Sodium fluoroacetate (Fluka A G) 3) Sodium malonate (Hopkin & Williams), 4) Sodium succinate (Hopkin & Williams), 5) Sodium fumarate (Fluka A G) 6) para Phenylenediamine (Riedel & de Haen) 7) 2 4 Dinitrophenol (E Merck A G), 8) Sodium citrate (E Merck A G)

Medium 199 containing 1000 mg glucose and 10 mg adenosine triphosphate (ATP) per liter from the media department Statens Seruminstitut Copenhagen was used throughout In some experiments the same medium without ATP was employed

Procedure employed in all experiments An experimental period of approximately 68 hrs was used Petri dishes each containing 4–5 million Hep-2 cells as confluent monolayers were inoculated with 10^5 TCD_{50} of RS virus giving a multiplicity of infection (m o i) of approximately 0 02 After an absorption period of 4 hrs maintenance medium was added and supplemented with one of the different compounds mentioned above At 20 44 and 68 hrs following infection (p i) the virus yield in the cell fraction and in the medium was determined as described previously (13) The Hep 2 cell cultures in the Petri dishes were in all experiments maintained in a 5 per cent CO_2 atmosphere at 35° C using approximately 0 17 per cent bicarbonate in the medium (13)

EXPERIMENTAL

RS virus is a comparatively slowly growing virus Even with a well adapted strain maximum titres are not reached until 40–50 hrs p i in the cell fraction with the m o i mentioned above With higher m o i maximum titres are reached at 36 hrs p i in Hep 2 cells (5) According to *Taylor-Robinson & Doggett* (19), who described a microplaque assay for this virus, there is no appearance of secondary microplaques in the tissue cultures before 70 hrs p i They employed approximately the same m o i as mentioned above This lends support to the theory that the effect of the added compounds in the experiments to be described is directed primarily towards the intra cellular fase of virus growth and presumably only in a minor extent towards the absorption and release of the virus from the cells

Effect of the number of cells infected on the virus yield Petri dishes containing three four, five and six million cells were each inoculated with 10^5 TCD_{50} of virus, and the virus yield determined in the cell fraction 20 44 and 68 hrs p i It was found that a doubling (or a halving) of the cell number at this m o i did not significantly influence the virus titre at these hours of harvest

Effect on cell count of the compounds employed in the experiments described below Table 1 contains a list of the different compounds (in maximum molar concentrations employed) whose effect on the virus yield has been examined The cell counts have been determined at the end of the experiments described below in 1) duplicate non infected Petri dishes containing the compound in question and 2) duplicate non

infected Petri dishes without the compound added (using a haematocytometer) It can be seen from the table that only sodium iodoacetate caused a major change in the cell count But even in the experiments with this compound, which had to be terminated at 46 hrs p i because of commencing degeneration of the cells, the change in cell number (less than 50 per cent) should, in accordance with the findings mentioned above, not be able to cause a significant alteration in the virus titre

TABLE 1

Effect on Cell Counts of Different Compounds Added to the Maintenance Medium

Compound added	Molar concentration employed	Cell count (%) after 68 hrs
p Phenylenediamine	4×10^{-4}	70 3*
2 4 Dinitrophenol	1×10^{-5}	103 9
Sodium citrate	1×10^{-2}	130 8
Sodium fumarate	5×10^{-2}	114 8
Sodium succinate	5×10^{-2}	102 8
Sodium fluoroacetate	$1 \times 10^{-}$[illegible]	89 0
Sodium malonate	$1 \times 10^{-}$[illegible]	123 3
Sodium iodoacetate	2×10^{-5}	56 0§

* Per cent of cell count in control cultures without compound added
§ Counted after 46 hrs

In the experiments to be described the virus titres given are the results of harvest of duplicate Petri dishes and in most cases the averages of repeated experiments

Effect of p-Phenylenediamine (pPD) According to *Greenstein* (12) pPD increases the oxygen consumption in tissue cultures by stimulating the function of the electron transport chain, although this reaction is less pronounced in tumor tissue than in normal tissue The function of the oxidative phosphorylation is dependent on the concentration of ATP being more active when the concentration is low (20) The effect of added pPD was therefore tested both in medium 199 without ATP and in normal 199

Employing normal 199 different amounts of pPD (10–50 µg per ml) were added to the maintenance medium in duplicate Petri dishes after absorption of the virus to the monolayers The cell fractions were harvested at 44 hrs p i and virus yield determined The added pPD did not affect the virus titres The same experiment was repeated using medium 199 without ATP as maintenance medium, but still no effect of the added pPD on the virus growth was observed The experiment using 199 without ATP was repeated, but the medium on the Hep-2 cell cultures was now changed three times over the last 48 hrs with medium 199 without ATP before inoculation with virus A reproducible effect of certain concentrations of pPD on the virus growth was now observed In Fig 1 is shown *the effect of different amounts of pPD added to the*

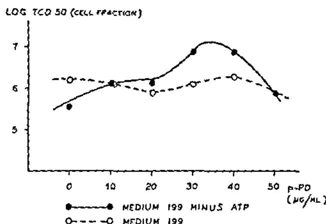


Fig 1

Virus titres obtained in cell fraction 44 hrs p i when different amounts of pPD were added to the maintenance medium

medium after absorption of the virus The dotted line shows the virus titres obtained in the cells 44 hrs p i when normal 199 was used, while the other curve shows analogous titres, when medium 199 without ATP was used and this medium had been used for changing the cultures three times during the last 48 hrs before inoculation with virus as mentioned above By supplementing the medium without ATP with 30 or 40 μg of pPD per ml the virus growth is now increased in the cells 44 hrs p i compared with the cultures to which no pPD had been added (Fig 1)

Fig 2 shows the virus titres obtained in the cell fraction and in the medium over an experimental period of 64 hrs In this experiment 199 without ATP was employed and the medium has been changed three

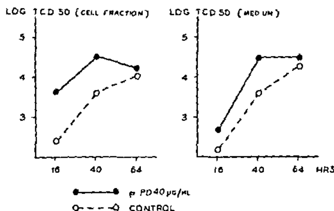


Fig 2

Virus titres obtained in cell fraction (graph to the left) and in medium (graph to the right) of cultures supplemented with 40 μg of pPD per ml of maintenance medium (199 minus ATP)

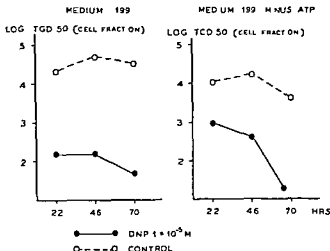


Fig 3
Virus titres obtained in cell fraction of cultures to which 10^{-5} M dinitrophenol was added to the maintenance medium (normal 199 graph to the left, 199 minus ATP graph to the right)

times during the last 48 hrs before inoculation It can be seen that the virus titres in the cultures supplemented with pPD (40 μg per ml) are increased in the first 48 hrs of the experimental period as compared with the control cultures to which no pPD was added This is mainly evident in the cell fraction

Effect of Dinitrophenol Fig 3 shows the effect of supplementing the maintenance medium with 10^{-5} M Dinitrophenol The virus titres reached in the cell fraction in cultures in which normal 199 was used are shown to the left in Fig 3 The graph to the right in Fig 3 shows analogous titres reached in cultures in which 199 without ATP was em-

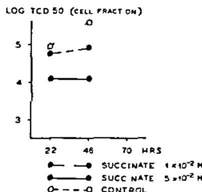


Fig 4
Virus titres obtained in cell fraction of cultures to which succinate was added to the maintenance medium in the concentrations indicated

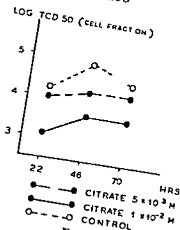


Fig 5

Virus titres obtained in cell fraction of cultures to which citrate was added to the maintenance medium in the concentrations indicated

ployed and used for 48 hrs before inoculation with virus as described above Dinitrophenol caused a marked depression of the virus titres as compared with the titres in the control cultures This effect is more pronounced in cultures in which normal 199 was employed

Effect of Tricarboxylic acids As described by *Krebs* (15), the addition of the intermediates of the tricarboxylic acid cycle to different animal tissues causes an increase in respiration in most of the tissues examined As is the case with pPD, this effect is less pronounced in tumor tissue than in normal tissues (6) Figs 4, 5, 6 and 7 show the virus titres obtained in tissue cultures in which the medium (normal 199) was supplemented with sodium succinate, sodium citrate or sodium fumarate as indicated Figs 4, 5 and 6 show the titres in the cell fraction of the harvested cultures, while Fig 7 shows the titres in the

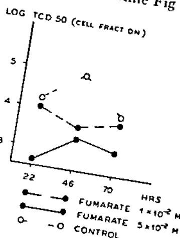


Fig 6

Virus titres obtained in cell fraction of cultures to which fumarate was added to the maintenance medium in the concentrations indicated

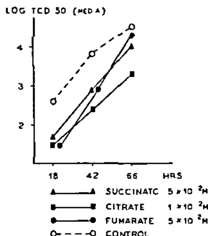


Fig 7

Virus titres obtained in medium of cultures to which succinate or citrate or fumarate were added to the maintenance medium in the concentrations indicated

medium from analogous experiments All the three different compounds caused inhibition of the virus growth in the cell fraction and in the medium when added in concentrations indicated in Fig 7 This inhibition was most pronounced during the first 48 hrs p i It must be stressed that a concentration of 5×10^{-2} M of fumarate caused a slight increase of pH in the cultures, so that this fact must be taken into consideration when the results are evaluated The experiments shown in Figs 4 5, 6 and 7 were repeated using 199 without ATP as maintenance medium

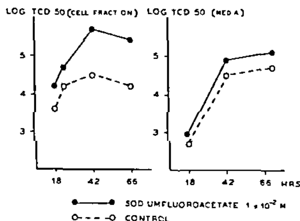


Fig 8

Virus titres obtained in cell fraction (graph to the left and in medium (graph to the right) of cultures to which fluoroacetate was added to the maintenance medium in the concentrations indicated

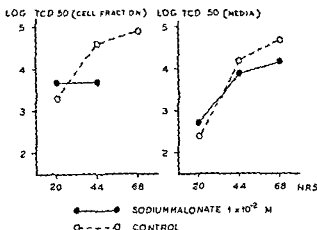


Fig 9

Virus titres obtained in cell fraction (graph to the left) and in medium (graph to the right) of cultures to which malonate was added to the maintenance medium in the concentrations indicated

but the results obtained were no different from those obtained when normal 199 was employed

Effect of Sodium fluoroacetate This compound inhibits the enzyme aconitase which promotes the oxidation of citric acid in the tricarboxylic acid cycle, and as a result of this inhibition there is accumulation of this acid Fig 8 shows the virus titres obtained in Hep-2 cell cultures in which sodium fluoroacetate was added to the maintenance medium (normal 199) in a concentration of 10^{-2} M No further change in virus growth could be obtained when 199 without ATP was used as maintenance medium When a higher concentration was tested the cells showed starting degeneration and a lower concentration had no effect on the virus growth in the assay system used Fig 8 indicates that the virus growth in the cell fraction was moderately increased by the added sodium fluoroacetate This finding was supported by an earlier appearance of the characteristic cytopathic effect (syncytia) in the cultures containing this compound The finding that no difference in titres in the media could be obtained might indicate that sodium fluoroacetate interfered with the release of the virus from the cells

Effect of Sodium malonate According to *Krebs* (15), when employed in a concentration of 10^{-2} M or less this compound only causes inhibition of the enzyme succinic acid dehydrogenase Fig 9 shows the virus titres reached in experiments in which the maintenance medium (normal 199) was supplemented with 10^{-2} M sodium malonate No effect of the added compound could be demonstrated As was the case with sodium fluoroactate a higher concentration of malonate caused degeneration of the cells Even with 10^{-2} malonate the cells showed commencing "rounding up" at 44 hrs p i The cell fraction titre could therefore not be determined after this time No definite difference of virus titres

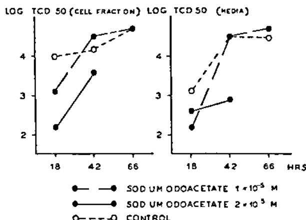


Fig 10

Virus titres obtained in cell fraction (graph to the left) and in medium (graph to the right) of cultures to which iodoacetate was added to the maintenance medium in the concentrations indicated

could be demonstrated on using 199 without ATP in the manner described above

Effect of Sodium iodoacetate This compound causes inhibition of triosephosphate dehydrogenases in the Emden Mayerhof pathway, but is it not considered to be very specific in its action It was added to the medium (normal 199) in the concentrations indicated in Fig 10 Only the higher concentration tested, 2×10^{-5} M, caused a depression of virus growth, as can be seen from Fig 10 As this concentration caused commencing degeneration of the Hep-2 cells after 42 hrs p i, the virus titres were not determined after this time

DISCUSSION

As described above, pPD caused an increase in the virus titre when added to the maintenance medium after absorption of the virus to the cultures If it is true that pPD stimulates the function of the mitochondrial electron transport chain (12) then this energy yielding mechanism seems to be of importance in the multiplication of RS virus in Hep-2 cells The rate of oxidation and production of ATP in the mitochondrial chain is determined by the concentration of intracellular adenosine disphosphate (ADP) (20) As the concentration of ADP increases, due to utilization and break down of ATP, oxidation in the chain increases With the purpose of increasing the concentration of ADP (relative to ATP) by omitting ATP from the maintenance medium experiments were carried out using medium 199 without ATP The finding that pPD stimulated virus growth only when this medium was used lends further support to the suggestion that the virus growth is dependent on the efficiency of the mitochondrial chain which might

possibly be more active and vulnerable in this medium The oxygen consumption of Hep-2 cells in Warburg flasks have been measured by the author in some preliminary experiments The cells were suspended in normal 199, tris-buffered, in a concentration of 3 million cells per ml *An addition of pPD (40 µg per ml) caused a 50 per cent increase of the* oxygen consumption during the first three hrs of the experimental period and a 100 per cent increase of the oxygen consumption during the following last two hrs of the experimental period as compared with the oxygen consumption of flasks to which no pPD was added

The importance of the oxidative phosphorylation for the RS virus synthesis is also supported by the results of the experiment, which revealed marked depression of the virus titres when the uncoupling agent DNP was added to the medium DNP was shown by *Ackermann & Johnson* (3) to inhibit the growth of influenza virus in chorion allantoic membranes (CAM) from embryonated eggs Similarly, *Eaton* (9) showed that DNP inhibited the growth of influenza virus and parotitis *virus in CAM in de-embryonated eggs On the other hand DNP did not* decrease production of polio virus in HeLa cells (11), but it has been shown that this virus can be propagated in HeLa cell cultures which *have been kept under anaerobic conditions* (10)

In the experiments described in this report citrate, fumarate and succinate inhibited the growth of RS virus only when added in concentrations which were relatively high compared with those which have been described as necessary for stimulation of oxygen consumption (15) Perhaps the fact that the Hep-2 cell line is a cancer cell line makes the use of higher concentrations necessary (6)

Ackermann (2) found that fluoroacetate decreased the growth of influenza virus in the mouse lung, but this result could not be reproduced by *Mogabgab & Horsfall* (17) Neither could these authors show any effect of this compound on the growth of influenza virus in the allantoic cavity of the embryonated egg (17) Fluoroacetate decreased the production of foot and mouth disease virus in bovine kidney cells (18) to a greater extent than malonate The lack of any inhibiting effect of fluoroacetate on the growth of RS virus in Hep-2 cells suggests that the normal functioning of the tricarboxylic acid cycle is not necessary for the multiplication of this virus This suggestion is supported by the finding that addition of malonate to the assay system employed similarly had no effect on virus growth According to *Ackermann* (1) addition of malonate to CAM, which was incubated in Warburg flasks and inoculated with influenza virus caused a decrease in both oxygen consumption and virus production The decrease was seen when malonate was added 4 hrs after the inoculation and also when malonate was added to the cultures together with the virus An inhibition of influenza virus growth in CAM by malonate was also demonstrated by *Eaton* (9)

In experiments reported by *Polatnick & Backrach* (18) inhibitors of glycolysis decreased the growth of foot and mouth disease virus in

bovine kidney cells more than did inhibitors of the tricarboxylic acid cycle In the experiments using RS virus described here iodoacetate, in contrast to the inhibitors of the tricarboxylic acid cycle inhibited the virus production Even though the effect of idioacetate is not considered very specific the results obtained suggest that glycolytic processes are of importance for multiplication of RS virus in Hep-2 cells If this suggestion is correct then aerobic glycolysis is more important than anaerobic glycolysis, as attempts to grow RS virus in Hep-2 cell cultures in an atmosphere of 5 per cent CO_2 in nitrogen caused a rapid decrease in the virus titre (unpublished results obtained by the author)

SUMMARY

Growth studies of RS virus in Hep-2 cell cultures with addition of different inhibitors or respiratory factors to the maintenance medium are described The results obtained suggest that the multiplication of this virus in the type of cell mentioned is less dependent on oxidative processes involving the tricarboxylic acid cycle than on other pathways of metabolism, perhaps glycolytic processes

It was possible to stimulate virus growth with paraphenylenediamine using medium 199 without ATP as maintenance medium Dinitrophenol markedly inhibited the virus growth Succinate, fumarate and citrate caused a moderate decrease in virus growth when added to the medium in certain concentrations Fluoroacetate and malonate did not cause *any inhibition of virus growth in the concentrations employed, in con* trast to iodoacetate, which had some inhibitory influence

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CLONE FORMATION IN TISSUE CULTURE

Experience from Long-Term Cultures of Irradiated Human Skin

By

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Received 30 iv 66

In order to investigate radiation induced chromosome aberrations in human cells, experiments have been designed in which human skin, exposed to radiation *in vivo*, has been studied after prolonged periods of tissue culture The subsequent chromosome analyses were then carried out upon cells taken from a series of subcultures The aim of the investigation has been to find out, whether or not, a quantitative relationship exists, between the dosage of radiation and chromosome aberrations after *in vivo* irradiation of human skin From the outset however, the study has been complicated by insufficient knowledge of a number of basic factors concerning the culture of cells damaged by irradiation Whether or not cells presenting radiation induced chromosome aberrations were able to live in tissue culture over prolonged periods and reproduce themselves, was not even known This did prove to be the case, and in the following paper some observations on clone formations derived from cells with certain radiation-induced chromosome aberrations, are described

MATERIAL AND METHODS

The material was taken from 4 patients aged between 31 and 42 years who had been admitted to the Radium Centre for irradiation of localized cancer Before carrying out the therapeutic irradation a trial dose was given to a circular area on the flexor surface of the right forearm of each patient The dosage used was 100 r for the first 3 patients and 500 r for the fourth The apparatus used in each case was a Muller RT 100 machine running at 100 kV 12 mA with a 1 7 mm Al filter Biopsies were taken from the first patient at 3 24, and 72 hours after irradiation and from the 3 others 24 hours after exposure Control biopsies were taken at the same times from corresponding areas on the patient s left fore arms

The biopsies were taken without local anaesthetic, by the method described by *Edwards* (2), which experience has shown to leave only a minimal scar

The 10 biopsies were treated in the following way (see *Frøland* (4)) Each was cut into small pieces These were placed at the bottom of four 25 cc Erlenmeyer flasks in a drop of cockerel plasma The flasks were then incubated for 1 2 hours after which the culture medium was added This consisted of 70 per cent medium 199 (Glaxo) 20 per cent pooled human plasma taken from pregnant women and 10 per

cent embryonic extract This culture fluid was changed 3 times a week half the contents of the flask being replaced each time After 2–3 weeks, when sufficient growth had taken place, the culture was trypsinized using a 0 01 per cent Trypure solution (Novo) Subcultures were now established, and these cultured in a similar way After about 3 days, the cells had usually grown sufficiently to cover the bottom of the flasks. At this stage some subcultures could be harvested, while others formed the basis for new subcultures Thus the process was continued until the required number of subcultures was obtained The subcultures were harvested on the day following a routine changing of the culture fluid, the only difference being that 4 drops of embryonic extract were also added A drop of a 0 04 per cent colcemid soltion (Ciba) was added and the whole then incubated for 4 hours, before being trypsinized The cells were next made hypotonic by the addition of 0 75 per cent sodium citrate solution and incubated for 15 minutes Finally the cells were fixed with a solution containing 3 parts methanol and 1 part glacial acetic acid

The chromosome slides were then prepared from the cell suspensions To do this the air drying technique described by *Rothfels & Siminovitch* (6) was used The chromosomes were stained with a Giemsa stain

The biopsies were cultured for 2–6 weeks The slides were prepared from a large number of subcultures as it was decided to take only one slide from each for analysis All slides were analysed 'blind" i e the observer did not know whether it originated from an irradiated or non irradiated piece of skin The aim was to analyse 150 cells from each skin biopsy

RESULTS

All the control cultures, togetehr with those derived from skin which had received 100 r, produced satisfactory growth This was not so with the cultures from skin which had been exposed to 500 r however, and after several weeks, further work on these was given up

The growth rate of the cultures seemed not to depend upon whether the biopsy was taken 3, 24 or 72 hours after irradiation Likewise there was no relation between the quantitative results, and the time of biopsy All the results will thus be considered together

TYPES OF ABERRATIONS

These may be divided into 3 groups abnormalities of chromosome numbers structural chromosome aberrations, and chromatid aberrations

Chromosome numbers About 10 per cent aneuploid cells were found, predominantly hypodiploids No statistically significant difference was found between cells from irradiated and non-irradiated skin

Structural Chromosome Aberrations Most of these were translocations, pericentric inversions and abnormal distribution between the sub-groups, into which the chromosomes are divided Only a single cell with a translocation was found amongst the controls Only a very few cells with deletions were observed These chromosome deletions should probably be regarded as "masked' reciprocal translocations No cells with dicentrics, tricentrics or ring chromosomes were found

Chromatid Aberrations Only a very few chromatid deletions or single fragments were found There were however a number of fractures and achromatic lesions, but the statistical difference between the irradiated and non-irradiated biopsies was insignificant

The structural chromosome aberrations can be divided into 2 groups stable and unstable The unstable aberrations include mainly chromosome deletions acentric fragments together with dicentrics tricentrics and ring chromosomes The description "unstable is used because it is expected that either the abnormal chromosomes or part of the chromosomes will be lost cause mechanical difficulties at mitosis (*e g* bridge formations) or for some other reason diminish the cell s chances of survival The stable aberrations include different types of translocations pericentric inversions and the abnormal distributions amongst the sub groups which can result from translocations These are referred to as stable aberrations as they are not expected to interfere with cell reproduction

In the present study statistically significant differences between irradiated and non irradiated skin were only found to occur with respect to the stable aberrations Amongst the 955 analysed cells derived from skin irradiated with 100 r 79 cells were found to contain aberrations These included translocations pericentric inversions and abnormal distributions amongst the sub groups following translocation In the 447 analysed cells from the controls only 1 was found to contain a stable aberration

It is difficult to discover whether or not cells with translocations of the types described above retain in culture the same potentialities for growth as do apparently normal cells The author has tried to elucidate this problem by relating the percentage of cells containing translocations to the time elapsing between biopsy and harvest of the subculture No definite relationship was found however The proportion of cells with translocations derived from the subcultures appears to be independent of the length of culture That is to say there does not seem to be any difference in growth ability after prolonged culture between apparently normal undamaged cells and those containing the translocations already described

Clone Formation The variation in the number of cells containing translocations in the cultures from irradiated skin was however considerable The spread was between 3 14 per cent of cells analysed from the different cultures The analysis as stated above took place blind and the preparations were also examined in random order Thus for some time it was not noticed that certain karyotypes with special abnormal chromosomes "marker chromosomes recurred in various subcultures Cells with such an identical karyotype must be regarded as originating from a single irradiated cell

Practically all cells containing chromosome aberrations must be assumed to originate from radiation damaged cells Therefore each cell with a specific aberration can be considered to be a member of a clone of which in most cases only one member is represented in the material The term clone has only been used in this study when the following conditions are satisfied a) at least 3 cells containing identical structural

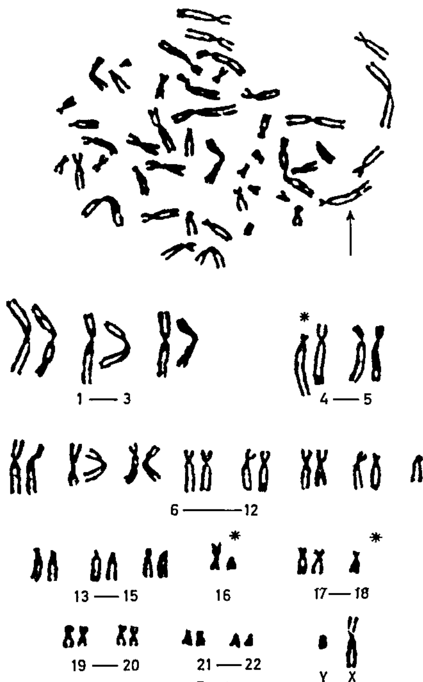


Fig 1

Culture II 674 of irradiated human skin Pericentric inversion of a chromosome in the 4–5 group Translocation between a chromosome No 16 and a chromosome in the 17 18 group resulting in abnormal numerical distribution between the 17 18 group and the 6 12 group Member of the same clone as the cell depicted in Fig 2

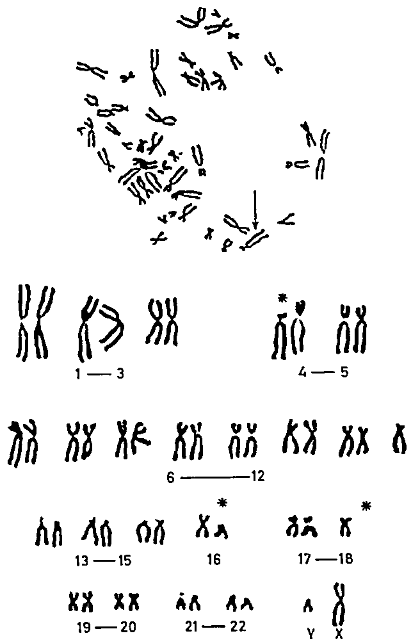


Fig 2

Culture II 674 of irradiated human skin Pericentric inversion of a chromosome in the 4 5 group Translocation between a chromosome No 16 and a chromosome in the 17 18 group resulting in abnormal numerical distribution between the 17 18 group and the 6 12 group Member of the same clone as the cell depicted in Fig 1

chromosome aberrations are found, b) other chromosome aberrations must not occur in the same cells, c) the cells must be found in subcultures all of which are derived from the same culture flask with the primary explant of the same skin biopsy Using this definition of a clone, 43 of the 79 cells with different types of translocations were found to come from 6 different clones, which could in turn be traced back to 6 radiation-damaged cells only The 6 clones formed 4 per cent, 2 per cent, 4 per cent, 15 per cent, 3 per cent, and 50 per cent respectively of cells in the possible subcultures The last number must be taken with some reservation however, since it concerned 6 of only 12 analysed cells

The manner in which the 6 clones influenced the large percentage variation of cells with translocations originating from skin irradiated with 100 r, was investigated Assuming that the clones are represented by only 1 cell each, both in the total number of analysed cells, and the total number of cells with translocations, it can be shown that the percentage of cells containing translocations is remarkably constant in the 5 cultures, and is within the expected statistical spread

Morphological study of the karyotype of the cells with radiation-induced chromosome aberrations, showed that the chromosome material was re-arranged in most cases There was no deficiency of chromosome material, neither was extra material present The karyotypes could thus be said to be "balanced"

This is illustrated in Figs 1 and 2

The 2 cells come from the largest clone in the material 18 cells in all were analysed, all of which were found to have identical karyotypes The cells were found in 4 subcultures all of which could be traced back to the same Erlenmeyer flask containing the primary explant from one definite skin biopsy The best explanation for the illustrated karyotype is that there is a *pericentric inversion on a chromosome in the 4–5* group together with a translocation of material from a chromosome No 16 to one in the 17–18 group

In conclusion it can be said that the radiation-damaged cells which contain abnormal chromosomes, and which can grow in the cultures used in this study, carry definite, stable aberrations which go to form balanced karyotypes

DISCUSSION

As stated above at the outset of the present study it was not known whether human cells with radiation induced chromosome aberrations could live and grow in our cultures It was commonly believed that the cells had to be analysed during the first mitosis after irradiation, because *many of the radiation-induced aberrations were supposed to be* cell-lethal, so that the cells in question do not survive the primary mitosis following irradiation, (see *Bender & Wolff* (1)) Presumably other less damaged cells probably succeeded in undergoing a few cell divisions *before* disappearing Since 1956 many studies have appeared concerning

abnormal karyotypes in man. Human pathology has shown that many types of chromosome aberrations are compatible with life of cells and individuals. We therefore thought it not unreasonable to expect that cells with certain *radiation induced* chromosome aberrations could reproduce themselves in our prolonged cultures.

Early in the present study clone formation was found to occur. This has been published in earlier reports (*Visfeldt* (8, 9)). Since then works from other sources have appeared which seem to support our findings although the experiments have been somewhat different from ours.

Goodman & Bender (5) have published the results of experiments in which heavily irradiated mice received transplants of either irradiated or non irradiated bone marrow. After a time clone formations of cells containing aberrations were found to be present in both groups. The karyotypes in the clone cells were not described more fully.

The findings of *Stroud et al* (7) are interesting. Cell cultures from pig kidneys were given 500 r and from this material some cell clones were isolated. These clones were studied at intervals over the following 3 years. The frequency of chromosome aberrations fell markedly to begin with but later a slow but constant increase in aberration frequency was seen over the 3 year period. *Most aberrations were exchanges resulting in markers that persisted and duplicated over many cell generations.*

The study which most closely resembles ours in which our results are referred to is that published by *Engel et al* (3). The authors investigated a case of chronic myeloid leukaemia developing in a man who 3 years previously received radio therapy following operation for bronchogenic carcinoma. An anterior and posterior field was irradiated and skin dose was 5280 r. At the 3 year follow up histological examination of the bone marrow revealed the presence of chronic myeloid leukaemia. Chromosome studies on the peripheral blood showed the Philadelphia chromosome to be present. Before beginning therapy a skin biopsy was taken from an irradiated area. From this material 4 cultures were set up. The cells were harvested after 26-36 days and 122 were analysed. 116 were diploid or near diploid and 6 were tetraploids or near tetraploids. Characteristic marker chromosomes which were found in 108 out of a total of 116 cells allowed classification under 5 well defined groups. The marker chromosomes described in the article are all, it seems, of similar types to those found in the present material, i.e. stable aberrations. The authors also state that unstable aberrations, acentric breaks, multicentric chromosomes etc. were not seen except in a single cell containing a ring chromosome. When many cells are found with the same characteristic chromosome aberrations the best explanation would seem to be that these cells originated from one definite stem-cell. In the authors' opinion therefore the 5 groups of cells represent a similar number of clones.

It is noteworthy that nearly all the cells analysed by *Engel et al*

belong to only 5 clones It is natural to assume that this reflects the actual state of affairs in the small skin biopsy which has been built up of a mosaic of cells originating from a very limited number of stem cells, which survived the massive irradiation received 3 years earlier

In my experiments the biopsies were taken so soon after irradiation that the cells can hardly have had time to divide *in vivo* Our clones are thus in principle different from those described by *Engel et al* They are derived from definite radiation damaged cells present in the primary explant and thus represent clones which have developed *in culture*

SUMMARY

The present investigation is intended as a contribution to the study of radiation-induced chromosome aberrations in human cells 3 persons received doses of 100 r on a small area of skin on the right fore-arm Skin biopsies were taken from the first patient 3, 24, and 72 hours after exposure, and from the other two, 24 hours after exposure At the same time, control biopsies were taken from corresponding un irradiated areas on the left fore-arm The biopsies were cultured for up to 6 weeks and chromosome preparations were made from a series of subcultures

Early in the investigation clone formation originating from cells with definite structural chromosome aberrations of stable types was found This observation of clones was important because it showed that cells with certain chromosome aberrations could survive and reproduce themselves in our cultures An analysis of clone cells and other cells in the material with structural chromosome abnormalities, suggests that one is dealing with balanced karyotypes

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ON THE PATHOLOGY OF ANGIOKERATOMA CORPORIS DIFFUSUM (FABRY)

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Angiokeratoma corporis diffusum (ACD) was first described by *Fabry* in 1898 (4) as a new entity, characterized by a peculiar skin rash Not until the autopsies of three brothers suffering from the disease were published by *Ruiter* and his collegues (15) did the real nature of the disease become known It was then recognized as a systemic disorder belonging to the lipidoses involving not only the skin but also various internal organs Since then a number of papers have been published on the disease, and it is now accepted as a lipidosis with intracellular accumulation of lipid, primarily in kidneys, heart, and vessel walls of probably all organs The lipid has until recently been accepted as a phospholipid (12, 20) However, in 1963 *Sweely & Klionsky* (18) showed that tissue from a patient dying from Fabry's disease contained two glycolipids consisting of ceramide + varying amounts of carbohydrate This links the disorder closely to the lipid disorder in Gaucher's disease (Table 1)

The present paper describes the pathology of seven patients belonging to three different families and includes four new autopsies A chemical analysis of tissue and serum from three of these patients will be published in another paper (1) The findings confirm those of *Sweely & Klionsky* and thus establish the disease as a disorder of the glycolipid metabolism

PATIENT A FAMILY I

(The case history and neurological findings of this patient have been described in details by *Jorgensen & Jorgensen* (12) and only a very short review of the complicated case history is given)

The patient was a 31 years old male He complained of being frail and tiring easily From the age of ten he suffered from a constant predominantly right sided headache

In late childhood—the exact date could not be determined—a skin rash appeared on the trunk in form of multiple small dark red, petecchial like spots These have persisted since then

As long as he could remeber he had severe pains in hands and feet when running a fever Apart from this he had shown no particular sensitivity to either heat or cold

At the age of twenty he had an attack of severe headache dizziness and diplopia accompanied by partial oculomotor paresis and diminished sensitivity of the left arm This attack showed an almost complete remission during the next months

TABLE 1

The Composition of the Lipids Involved in the Known Lipidoses

Composition	*Name*	*Disease*
H H H $CH_3(CH_2)_{12}$–C C C–C–CH OH H OH NH_2	Sphingosine	
Sphingosine + Fatty Acid	Ceramide	
Ceramide + Phosphoric Acid + Choline	Sphingomyelin	Niemann Pick
Ceramide + Hexose	Cerebroside	Gaucher
Ceramide + Hexose + Hexose + Hexose Ceramide + Hexose + Hexose		Fabry
Ceramide + Hexose + Hexose + Hexosamine Sialic Acid	Ganglioside M_2	Tay Sach
Ceramide + Hexose + Sulphate		Metachromatic Leucodystrophy

About five years later at the age of 25 he had a similar, but much more serious attack and was admitted to a neurological ward This attack started with violent headache followed by partial right sided hemiplegia and difficulty of speech He showed mild central paresis of the left facial nerve and some spasticity of the right arm and leg These signs cleared up almost completely during the subsequent weeks but reappeared with paresis of the left arm and facial nerve accompanied by fever of 39 6° C These symptoms regressed somewhat but signs of definite neurological lesions remained The speech remained slow and slurred and he had oculomotor instability with nystagmus and paresis of the left facial nerve There remained *slight weakness on extension of the right elbow and hyperactive tendon reflexes on* both sides combined with bilateral ataxia and dysdiadochokinesia Both legs showed spasticity

A few months later he suddenly developed a massive left sided hemiplegia which since then has remained unchanged

Shortly after this he had a series of transient attacks of choreo athetoid movements of the right arm each lasting for a few minutes These attacks subsided but left complete loss of speech

During his stay at the hospital biopsies of kidney and skin were performed

Apart from the neu
On the lateral aspects
genitals and the sacr
neck and extremities
caruncles and along the vessels on the scleral conjunctivae The lesions varied in size from the minutest dust like, red spots to 2 3 mm large slightly elevated darker purplished elements of a rounded shape showing a symmetrical destribution and a typical morphology

Ophthalmoscopic examination showed another typical finding with dilated, tortuous veins in the ocular fundi

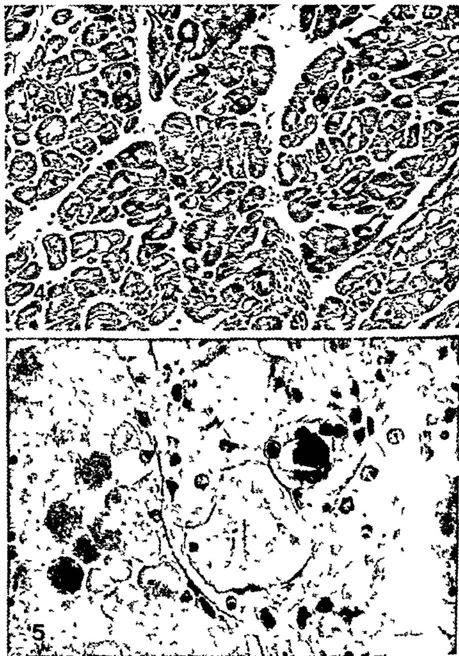
Apart from these findings the patient showed a constant proteinuria of about 500 mg a day but normal kidney function

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Figs 1 2

Fig 1 First kidney biopsy from patient A family I The glomerular epithelial cells are large with foamy cytoplasm PAS stain × 525

Fig 2 Same biops as showed in Fig 1 The tubular epithelium contains foam cells PAS stain × 525



Figs 4 5

Fig 4 Heart muscle from patient A family II The muscle fibres are swollen with vacuoles and ring structures H & E stain × 84

Fig 5 Patient A family II kidney tubulus with numerous lipid laden macrophages and a homogenous small cylinder PAS stain × 525

Autopsy

External examination No angiomatas were visible after death presumably due to terminal collapse

Apart from these findings the macroscopical examination showed nothing of special interest No enlargement of the spleen or lymph nodes was seen

By microscopical examination prononunced changes were found in heart kidneys spleen liver and brain All myocardial cells showed severe damage They were almost all enlarged on transverse section and many of them had a central apparently empty vacuole Some cells showed displacement of the nucleus and a ring like structure PAS stain showed a few bright red large macrophages in the interstitial tissue and a faintly PAS positive substance in the vacuoles of a few of the muscle cells (Fig 4) Staining with Sudan Black was negative, and the vessels presented no detectable changes

The kidneys were severely damaged Only a few glomeruli appeared normal most showed a large glomerular tuft with blood filled dilated capillaries The basement membrane was normal in most of the glomeruli but slightly thickened in others and in some it presented large lumpy, strongly PAS positive deposits In a few of the glomeruli the tuft was changed into a solid hyaline PAS positive mass Only very few epithelial cells in a few scattered glomeruli showed the foamy appearance seen in the previous patient A few macrophages were found in the capsular space The tubuli and collective ducts showed scattered homogenous PAS positive cylinders but no epithelial foam cells In a number of tubuli were found numerous large rounded macrophages with an abundant, foamy cytoplasm which stained strongly with Sudan Black and PAS stains (Fig 5)

The vessels showed pronounced changes In arteries of all sizes the media was thickened from intracellular depositions of a lumpy substance in the muscle cells strongly positive to PAS and Sudan Black stains Fresh thrombosis were seen in some of the smaller arteries The intima was not involved (Fig 6)

The spleen showed changes in the arterial walls similar to those of the kidneys The red pulp was normal with no signs of reticulosis A very few PAS positive macrophages were scattered throughout the tissue

The liver showed signs of chronic stasis but was without detectable changes in the arteries of the periportal spaces Some large PAS positive macrophages were found scattered in the liver tissue both in the periportal spaces and the sinusoids

The brain[1] showed severe cellular damage subsequent to anoxia and severe brain edema The smaller arteries had amorphous intracellular deposits in the muscle cells of the media similar to those found in the other organs The deposits were most pronounced in the region of the corpus striatum and stained with PAS and Sudan Black Distinct changes were found in the ganglia cells of nucleus & supra opticus preopticus, paraventricularis, and oliva In these nuclei the ganglia cells appeared markedly swollen with large cytoplasmatic vacuoles containing a finely granular substance which stained with PAS and Sudan Black with varying intensity, most strongly in the cells of oliva Also the ganglia cells of formatio reticularis and nucleus dorsalis n vagi appeared swollen but without inclusions Tissue samples from kidney heart the cerebral cortex and from globus pallidus were subjected to chemical analysis (1) which showed abnormally high contents of glycolipids identical to those found in tissue from patient A family I

FAMILY III

Four brothers belonging to this family all died from uremia in their middle thirties The disease was proven by autopsy in three of the four cases The fourth brother probably died from the same disease although definite proof is lacking

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[1] The examinations of the brain was performed by Dr *Erna Christensen* University Institute of Neuropathology



Figs 6 7

Fig 6 Patient A family II kidney arteries Deposits in the cells of the media of arteries of varying sizes PAS stain × 376

Fig 7. Patient family III kidney with severe damage, all glomeruli almost totally destroyed PAS stain × 52

Patient A

The patient a 37 years old male was admitted to hospital for the first time a'

Seven years later he was admitted to an ear nose throat service because of fever Proteinuria was now present and the BSR was markedly increased to 129 132 mm hour The patient complained of fatigue and lumbar pains and the painful attacks in hands and feet still persisted His kidney function was normal The skin was normal He was next admitted to a medical ward nine years later 33 years old His *predominant complaint was now fatigue and somnolence Paresthes as in fingers* and toes now accompanied the attacks in hands and feet The kidney damage had progressed as urea clearance was now 39 48 ml/min proteinuria was 5–7 g/liter and the blood pressure was 170/70 He was admitted to a medical ward for the las' time in 1963 in a state of uremia Serum urea was 303 mg% serum creatinine 22 4 20 2 mg/100 ml clearance 2 ml/min Blood pressure was normal Ophthalmoscopic examination showed abnormally dilated and tortuous veins

The patient died in uremia 37 years old The clinical course was dominated by renal failure with only vague neurological symptoms in the form of painful attacks in hands and feet

Autopsy

External examination The skin appeared normal

Internal examination The lungs showed pronounced edema The heart was moderately enlarged weighing 490 g The myocardium was of a uniform light brown colour and markedly friable The kidneys were slightly diminished in size pale and very soft but otherwise normal The macroscopic autopsy showed nothing else of interest

Microscopical Examination

Kidneys Almost all glomeruli were completely destroyed The glomerular tufts formed shrunken homogenous masses and many glomerular capsules appeared [illegible]

collective tubules contained similar large macrophages In some areas tubuli showed atrophy The epithelial cells appeared large many of them with abundant light cytoplasm The interstitial tissue showed fibrosis and a few lymphocytes and foam cells were present

The kidney arteries of all sizes showed pronounced and characteristic changes Abundant masses were deposited in lumps or more diffusely in the muscle cells of the thickened media The intima appeared unchanged or only slightly thickened and was without deposits (Figs 10 and 11)

Similar vascular changes were found in spleen suprarenal glands myocardium and brain

The myocardium showed pronounced changes similar to those of the previous patient Almost all cells were swollen many with a central vacuole producing a ring like appearance The vacuoles although usually appearing empty contained in some cases a granular substance

The lymph nodes showed reticulosis with broad bands of large light reticular cells with abundant cytoplasm

The substance in vessel walls in macrophages in epithelial cells in glomeruli and tubuli was *stained bright red by PAS stain and black with Sudan Black Other lipid* stains as osmic acid and Sudan III gave negative results The staining reactions were in agreement with the hypothesis that a glycolipid was present Staining reactions for amyloid and glycogen were negative

Examination of the brain[1] showed moderate acute and chronic degeneration of

[1] The brain examination was done by Dr *Erna Christensen*





Figs 8, 9

Fig 8 Patient A, family III. Glomerulus with foamy cytoplasm in epithelial cells and macrophages. PAS stain × 525

Fig 9 Patient A, family III, kidney. Two glomeruli with shrunken glomerular tuft and lipid laden macrophages in capsular space and tubuli. PAS stain × 210



Figs 10 11

Fig 10 Patient A family III kidney with deposits in arterial wall and macrophages in capsular space Sudan Black × 273

Fig 11 Patient A family III kidney artery with deposits in the muscle cells of the media PAS stain × 525





Figs 8 9

Fig 8 Patient A, family III Glomerulus with foamy cytoplasm in epithelial cells and macrophages PAS stain × 525

Fig 9 Patient A, family III Kidney Two glomeruli with shrunken glomerular tuft and lipid laden macrophages in capsular space and tubuli PAS stain × 210

No autopsy was performed

It appears from informations obtained from his brothers that his symptoms and case history were closely similar to theirs and it seems safe to assume that his disease was of the same nature

COMMENTS

Seven cases of Fabry's disease (ACD) are presented Two of these are still alive (patient A and B family I) and the diagnosis in these two cases is based on a typical history on vascular anomalies and on chemical analysis of kidney biopsies and serum and urine from both

In two cases (patient A family II and patient A family III) the diagnosis is obtained both by autopsy and by chemical analysis of tissue

In addition three more cases are presented of which two (patient B and C family III) exhibited typical histories and findings at autopsy and a third (patient D family III), in which the diagnosis is only tentative but fairly certain

The clinical manifestations and symptoms of the disease are extremely varied The symptomatology however is thought outside the scope of the present paper *Wise et al* (22) have given a thorough survey of both the history of the disease and its symptomatology to which the reader is referred

The disease is hereditary with strong preponderance and poor prognosis in the male although females may show the disease in a weaker form Its mode of inheritance is dealt with in the paper by *Wise et al* (22) and *Gemmingen et al* (7)

The pathological changes in these patients confirm the concept of ACD as a storage disease as first postulated by *Ruiter* and coworkers in 1947 (13) It has generally been considered a lipidosis and the substance deposited has since *Scriba's* paper (17) been taken as a phospholipid and has been accepted as such by a number of subsequent authors

The chemical analysis performed on tissue blood and urine from four of our patients however has shown the deposits to be glycolipids consisting of one molecule ceramide with two and three molecules of hexose It is thus closely related to the lipid found in Gaucher's disease which consists of one molecule ceramide and one molecule of hexose *These findings thus confirm those of Sweely & Klionsky (18)*

Pathological Findings

The patients with ACD show wide-spread and severe lesions of the blood vascular system This damage takes different forms

The vascular lesions in the skin have been known from the original patient described in biopsy preparation by *Fabry* in 1898 (4) and from later descriptions by *Pompen et al* (15) and others The lesions consist of scattered thinwalled dilatations of v in ing very close under the

epidermis but without vascular proliferation Thus they have the character of phlebectasias rather than true angiomatas The size of these dilatations vary from tiny hardly visible spots to prominent nodules 2 3 mm in size containing dark red blood The most superficial ones are limited by a single layer of endothelial cells The typical distribution of the skin rash is found in patient A and B family I It is of interest that the degree of skin involvement varies from patient to patient The skin rash was thus less prominent in patient A than in patient B The skin rash in patient A family II was only moderately developed although typical whereas the four brothers in family III showed no or almost no lesions at all or lesions that were hardly discerned and which played no role at all in the clinical picture Other patients without discernible skin lesions have been described by *Hamburger* (8)

Also the superficial vessels in the eye show typical changes *Weicksel* (21) noted that the veins in the conjunctiva and retina were abnormally tortuous and dilated

These conjunctival vessel changes are illustrated by Fig 3 from patient B family I The veins show dilatation with uneven calibre and series of microaneurysms these probably corresponding to the phlebectasias in the skin

The retinal changes have been observed best in patients A B C family III and in patient A family II in whom also slight opacity of the cornea was seen

Although these vessel changes are important for diagnostic purposes they do not seem to play any role in the serious manifestations of the disease which probably can be explained by the changes in the arterial walls

This change which was first described by *Ruiter* and coworkers (15) *in connection with the first two autopsies done on a patient with* ACD consists in intracellular deposits in the muscle cells of the media It has been shown to affect the smaller arteries and the arterioles but apparently not the larger vessels The deposits increasing the thickness of the arterial walls leads to a narrowing of the lumen The interna is not affected These changes have been found in all organs and are thus the manifestation of a systemic order

Arterial lesions are also found in skin biopsies which are therefore useful for making the diagnosis These deposits stain with PAS and Sudan Black and are birefringent (11)

In the present material the smaller arteries in the kidneys show these changes most strongly It can be assumed that the thickening of the arterial walls and narrowing of the lumen may explain some of the vague and varied neurological symptoms that many of these patients show These symptoms tend to show a characteristic pattern of peripheral pain and vasomotor disturbances but different symptoms may be seen as appears from the history of patients A B and C family III

The early severe and acute cerebral damage which in this material is seen in patient A family I is well known from the literature (22 18) This may be explained by thrombosis of the affected vessels It may in this respect be noted that also the smaller arteries in the kidney from patient A family II showed fresh thrombosis

Scriba (17) and *Rahman & Lindenberg* (16) have published detailed neuropathological investigations on the central and peripheral nervous system from two patients with ACD They report the same vessel changes and lipid laden macrophages in the central nervous system as seen in the present two autopsies In addition to this they have reported ganglia cell changes with ballooning of the cells with foamy birefringent deposits in the cytoplasm These abnormal ganglia cells were found to have characteristic distribution in the brain namely in the supraoptic paraventricular and preoptic ganglia of the hypothalamus isolated cells in the tuber cinerum nucleus amygdalae basalis and substantia nigra In the cortex only the subiculum of hippocampus contained cells In addition to these cells abnormal swollen ganglia cells were found in the peripheral sympathetic ganglias and the plexus myentericus in the gastrointestinal tract

Lesions in these centers may well explain the striking autonomic disturbances displayed by these patients In the present material both brains examined showed these changes The ganglia cells in the locations mentioned showed ballooning in varying degrees and cytoplasmatic inclusions most of which stained with PAS and Sudan Black The other signs of damage seen in the two brains examined the edema and non specific cell degeneration may be explained as secondary to other factors such as uremia severe shock and anoxia However, control sections from the same ganglias from patients with diseases other than ACD did show varying degrees of the same changes with ballooning and accumulations of a granular material in the ganglial cells The significance of these changes therefore seems uncertain and must await further clarification

It is of special interest to note that whatever changes found—both in the present material and by the previous authors—only involve the connective tissue of the brain the vessel walls some macrophages and possibly *some specific nerve centres belonging to the autonomic system*

Thus ACD differs distinctly from the other lipidosis affecting the brain This distinction has also been noted by *Rahman*

Kidney Pathology

A prominent trait in the pathology of ACD are the kidney lesions which led to total uremia in the majority of the published cases The present material shows a progression of these changes from patient A family I in which the damage is judged by two subsequent kidney biopsies through patient A family II wh e disease was dominated by

cardial insufficiency, but who did show definite signs of renal failure to the three patients in family III, who all died in frank uremia

The earliest changes were observed in kidney biopsy from patient A family I showing foamy swelling and vacuolisation of the epithelial cells in the glomerular vessel tuft and tubuli These changes correspond closely to what is described by earlier authors also on the basis of kidney biopsies (2, 10, 9, 19, 14, 20, 6, 8) These cytoplasmatic inclusions presumably represent accumulations of the abnormal glycolipids which can also be demonstrated in the very large number of laden macrophages seen in the capsular space, tubuli, and collecting ducts These macrophages form a convenient criterium of the disease as they are readily recognized in the urine (19) The substance is very strongly, but unevenly stained with PAS and Sudan Black in the later stages in the disease, but not in the earlier ones The reason for this is unknown Examination under the electron microscope has shown deposits of a strongly electrondense lamellated substance intracellularly both in the endothelial and epithelial cells (10, 9, 14, 8) Although the staining properties of the lesion in the early stages of the disease, when a kidney biopsy would be especially useful are uncertain, the morphological changes are very typical and easily recognized

As the kidney lesion progresses, both glomeruli and tubuli show increasing destruction As the renal architecture is retained with no or slight interstitial fibrosis the changes are due presumably to epithelial lesions caused by the abnormal deposits rather than to consequences of the arterial lesions

In the late stages many vascular tufts have completely disappeared and the capsular spaces appear empty, others are converted into solid shrunken masses, which are strongly PAS positive In a few glomeruli the characteristic honey comb appearance of the epithelial cells can however, still be recognized In these late stages the macrophages in the tubuli and the arterial changes are dominant

These kidney changes are thus in all stages unique and readily recognizable and do not show any resemblance to any other kidney affliction

Cardiac Lesions

Another characteristic trait in the pathology of ACD is the damage of the myocardium In all three earlier autopsies described (15, 17) an enlargement of the heart and characteristic microscopical changes of the myocardial fibres have been found See also (13) The heart weight may be markedly increased In two cases (15), one heart weighed 450 g, and the other 615 g, in Scriba's case the weight was 550 g In the present material the heart weights were 490 g, 530 g, and 730 g from the three brothers in family III In the patient of family II, whose clinical condition was dominated by cardial insufficiency the heart

weighed 760 g All hearts examined showed a peculiar friable con sistency

Microscopically the lesion consists of swelling of the myocardial muscle with irregularity and polymorphism of the nuclei Almost all cells display a large central vacuole which in most cases appear empty giving the cells a ring like appearance In a few cells however it stained a deep red with PAS indicating probably the presence of the substance found in vessel walls and in the renal parenchymal cells Why the vacuoles appear empty in the majority of cells remain unexplained *Pompen et al* offer the hypothesis that it is caused by a substance in a fluid state The changes in arterial walls of the interstitium described by earlier workers have not been seen in the present material This may be fortuitous as the arterial changes are probably systemic

The lesions of the myocardium seem to give remarkably few symp toms It appears from the literature that many patients show enlarge ment of the heart and electrocardiographic changes but very few show cardial insufficiency In the present material signs of clinical heart involvement were lacking in the four patients in family III although the hearts at autopsy were found grossly enlarged indeed in patient B family III it weighed 730 g

In the patient from family II with severe cardial insufficiency the heart showed the most severe lesions the weight being 760 g and the histological changes most pronounced

Spleen and Liver

In contrast to the classical lipidoses liver and spleen show only scant changes

The spleens described by the earlier workers have shown changes in the arterial walls as elsewhere in the body and some deposits of iron pigment

In the present material arterial lesions were seen but otherwise no significant changes In the patient from family II a few scattered large macrophages were found in the periportal spaces and sinusoids These cells were laden with PAS positive material whereas the liver cells proper were unaffected

A lymph node from patient A family III showed moderate reticulosis Some but only a minority of the reticular cells were swollen and filled with PAS positive material

Apart from these changes a detailed microscopical examination of all organs did not show changes of interest to the present topic No foam cells were found in the bone marrow These have been described by other authors (5 20) They take the form of scattered isolated lipid laden macrophages of the same shape and staining properties as those found in the present material in the interstitial tissue in the liver heart spleen and lymph nodes No changes of the bone marrow as in Gaucher s disease have been described

The general pathological picture thus differs sharply from that of the classical lipidoses The glycolipid found in ACD is in chemical composition quite close to that found in Gaucher s disease differing only in the amount of hexose in the molecule *In spite of this both the* clinical course the macroscopic and microscopic pathology and the morphology of the single lipid laden cell are quite different in the two diseases In ACD is found none of the enlargements of the spleen or liver or the bone marrow and skeletal lesions so typical of the adult variety of Gaucher s disease

Niemann Pick s disease the other classical lipidosis also displays a clinical and pathological picture which is quite distinct from that of ACD *In this respect it is striking that general ganglia cell involvement* which is so typical of Niemann Pick s disease is not found in ACD

CONCLUSION

It can thus be concluded that the present material consisting of seven cases of ACD including four autopsies confirms the concept of the disease as a systemic disorder belonging to the lipidoses The lipid has been found to be glycolipid and to be identical in samples from three patients belonging to three different families The case histories have demonstrated the variability of the symptoms All patients *displayed* some degree of renal damage which in three (probably four) patients from family III dominated the course and finally led to death in uremia *The course of the disease in two other patients was dominated by* neurological damage and cardial insufficiency the remaining patient from family I being as yet without any severe symptoms The skin affliction which had led to detection of the disease may vary much in degree and may even be absent The relation between the abnormalities *of the skin vessel and the internal lesions representing the storage* disease remains unexplained as does the nature of the basic metabolic disturbance

SUMMARY

The material presented comprises three families containing in *all seven* cases of ACD The case histories are presented and the *pathological* findings described in details The very polymorphous symptomatology *with dominance of either neurological cardial or renal* symptoms is shown Renal damage is always present whereas the skin lesion which originally led to the detection of the disease may be absent *The* material shows the very distinct unique pathological changes which are pathognomonic for angiokeratoma corporis diffusum in various *degrees of severity*

Corresponding chemical analysis on tissue from kidney biopsy from family I and from two autopsies in the two other families shows the abnormal accumulation to consist of glycolipid The chemical com

position is that of ceramide plus two or three molecules of hexose This links the metabolic disorder close to that of Gaucher s disease In contrast to the chemical findings the clinical course and the pathology are quite distinct in the two diseases

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A BIOCHEMICAL INVESTIGATION OF ANGIOKERATOMA CORPORIS DIFFUSUM

By

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Received 28 iii 66

The first chemical interpretation of material deposited in angiokeratoma corporis diffusum (ACD), or Fabry's disease, was suggested by *Kuhnau* (1950) cited in the article of Scriba (11) The material was suggested to be a diaminophosphatide, closely related to sphingomyelin However, in 1963 Sweeley and Klionsky (13) published a biochemical study of kidney tissue from a 28 year old man with Fabry's disease who died of renal failure The kidney contained about 15 mg per g of wet tissue of a trihexose ceramide (TCH) with the structure ceramide glucose-(galactose)$_2$ This compound could not be demonstrated in normal kidney tissue Furthermore, smaller amounts of a dihexose ceramide (DHC) with the structure ceramide-(galactose)$_2$ were present

Quite simultaneously the occurrence of THC with the same carbohydrate sequence was demonstrated in normal spleen by *Makita & Yamakawa* (6), in serum by *Svennerholm & Svennerholm* (14), and also in normal kidney in small concentration by *Mårtensson* (8) The latter investigator was able to isolate 115 mg monohexosides, 223 mg dihexosides, 568 mg trihexosides and 1003 mg aminoglycolipids from 542 g dry kidney tissue normal kidney tissue, or 0,04, 0,08 0 2 and 0 4 mg per g respectively *Mårtensson* (8) also demonstrated that the THC in normal kidney did contain the same carbohydrate sequence and had the same general pattern of fatty acids as the material isolated by *Sweeley & Klionsky* (13) from a Fabry kidney

By the methods used in the present communication it has been possible to confirm the findings of *Sweeley & Klionsky* that DHC and THC are deposited in the kidney in Fabry's disease Furthermore it has been established that they are found in high concentrations in other tissues as well It has also been possible to evaluate quantitatively and semiquantitatively the content of THC in well defined regions in brain, heart, and kidney as well as in serum and, tentatively, in urinary sediment *in four cases of Fabry's disease from three different families* The aim has been to present simple methods which can be used in clinical screening

PATIENT MATERIAL

Organ Material

Fam ly I patient A renal b opsy specimen (5 mg wet weight primarily medullary tissue)

Family II patient A kidney cortex and medulla heart muscle brain frontal c rtex corona radiata and globus pallidus

These specimens were obtained at autopsy within 16 hours after death and were formaldehyde fixed for 24 hours before biochemical study

Family III patient A kidney cortex and medulla (Formaldehyde fixed for one week before study

Serum and Urinary Sediment

Family I patient A patient B
Family II 2 sons of patient B
Family II 3 sisters of patient A and four of their children

Blood was obtained by venous puncture and afterwards serum was immediately subm tted to the extracti n procedure Urinary sed ment was obtained by low spee l centrifugation (2000 G) of morning urine

Normal control material of the same organs was obtained from 5 autopsies of pat ents in the age group between 30 and 50 years and serum and urinary sediment from 10 normal persons under the same cond tions as mentioned above

The clinical and pathol gical data of the patients will be published elsewhere (5)

METHODS

Lip d Extraction

filtered thr ugh a prewashed filter paper

Isolation of Sphingol p ds

1 5 vol of an aquous KOH solution was added to the lipid extract to reach a final concentration f 0 5 equivalents of KOH The m xture was shaken at 37° C for 16 hours under nitrogen aci lified to pH 4 with HCl and allowed to stand for 1 hour The lipid was then extracte l 2 times with equal volumes of chloroform in a separa tory funnel The chloroform phases were combined and evaporated to dryness at 50° in an atmosphere of nitrogen The residue was red ssolved in chloroform and submitted to analytical preparative or quantitative thin layer chromatography (TLC) In this way a quantitative recovery of the glyco sphing l pids and a com plete saponification of the glycerolipids was obtained

Analyt cal and Preparat ve TLC

These pr ce lures were performed as previ usly described (2 3)

Chem cal Characteri at on f Isolate l Glycol p ds

1) Element analysis of SCN an l H was kin lly perf rmed by Preben Hansen Microanalytical Department H C Orstedsinstituttet Un versity f Copenhagen l was determine l acc rd ng t *Svanborg & Svennerholm* (12)

2) Analysis f carl ohydrates was perf rmed by paper chr matography after pr l nged r partial hydrolysis of glyc lipi ls Pr panol water (4 1) was used as de vel ping system The hydrolysates were applie l as sp ts on Whatman paper N 3 together with known carbohydrate standar ls an l the chormatogram develope l for 3 hours in a saturated chamb er with the d mens ons 20 25 20 cm After development the spots were detected by spraying with anil n phtalate accord ng to *Schult e Schmidberger & Haupt* (10) For detection f t tal carb hydrates a prolonged hydrolysis was p rf rmed before chr ma ography F the chloroform methan l extract of the sp t fr m preparative TLC wa ad l l n equal v lume f 5 N HCl,

and the mixture boiled under reflux for 4 hours The chloroform phase was allowed to evaporate and the aqueous phase kept in an electrical oven at 90° for an additional 4 hours and finally concentrated in vacuo at 50° In order to elucidate the sequence of carbohydrates in the glycolipid molecule the chloroform methanol extract of the spot from preparative TLC was submitted to partial hydrolysis an equal volume of 5 N HCl was added and the mixture boiled under reflux for 30 min then the phases were separated in a separatory funnel and the aqueous phase concentrated for paper chromatography as mentioned above The partially degraded glycolipids in the chloroform phase were then isolated by means of preparative TLC and submitted to prolonged acid hydrolysis for determination of residual carbohydrate moieties

3) Gas liquid chromatography of fatty acid methyl esters was performed after methanolysis of glycolipid using H^+ as a catalyst The isolated glycolipid was dissolved in a mixture of 5 per cent sulphuric acid in anhydrous methanol and heated to 100° for 8 hours in sealed tubes Then the methyl esters were extracted with hexane in a separatory funnel and injected into a Perkin Elmer gas chromotograph model 116 E using a standard diethylene glycol succinate polyester column operated at 214° argon as a carrier gas and a flame ionization detector The peaks were identified by injecting known methyl esters as standards and by comparing with Rf values compiled from the literature (1) The area of each peak was regarded as directly proportional to the amount of methyl ester eluted in the peak (1)

Infra Red Spectroscopic Characterization of Isolated Glycolipids

The isolated glycolipids were dissolved in chloroform and evaporated on a KBr disc made of 100 mg KBr in a Unicam evacuable die mill (MK III) under 25 tons per cm pressure and 15 mm Hg vacuum Then the glycolipid applied on the KBr disc was analysed in a Unicam SP 200 infra red spectrophotometer The spectrum was analysed as previously described (2 3) using the law of group frequences

Quantitative Estimation of THC

Known amounts of THC isolated by preparative TLC as well as lecithin obtained from BDH Ltd was applied on 2 cm long straight lines at the line of origin on a

tance from origin at the chromatoplate) was found to be proportional to the amount of glycolipid present in the range studied (0 150 μg) and the ratio $\frac{\int E_{m_0\,THC}\,dt}{\int E_{m_0\,lecithin}\,dt}$ was found to be 0 40 In experiments purpoting to determine the concentration of THC a known lecithin standard (m_0) was run on the same chromatoplate and the amount of THC present calculated as $\frac{\int E_{m\,THC}\,dt}{\int E_{m_0\,lecithin}\,dt}\ m_0\ 0\ 4$ The standard deviation $s = \sqrt{\frac{\Sigma(a\ a)^2}{n\ 1}}$ was found to be 7 5 per cent based on seven experiments

RESULTS

Organ Material

Analytical TLC TLC of sphingolipids from the pathological material studied demonstrated in all cases the presence of two fractions THC and DHC, with Rf values of 0 43 and 0 62 respectively in the neutral solvent system (chloroform methanol water in the proportion 70 30 4) (Fig 1, 2, and 3) Both fractions failed to stain violet with

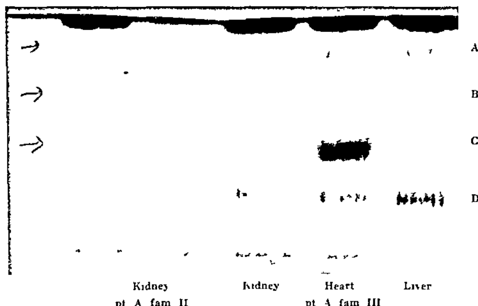


Fig 1

Thin layer chromatogram of sphingolopids isolated by saponification from two patients with Fabry s disease Devel ped with chloroform methanol water 14 6 1 sprayed with amonium molybdate perchloric acid reagent A cerebrosides B dihexose ceramide C trihexose ceramide D sphingomyelin and aminoglycolipids Fraction B and C can not demonstrated in normal tissue by this meth od

TABLE 1

Flement analysis of THC analytical values (Source of origin heart case A family II) %		Values calculated from formula %
P	0 00	0 00
S	0 00	0 00
N	1 81	1 23
H	9 94	9 99
C	60 65	6? 13
O	27 60	26 6?
	100 00	100 00

* Calculated by substraction of the sum of the percentages of the other elements from 100 00

orcinol HCl reagent for sialic acid, but stained with diphenylamine for carbohydrate

It was not possible in any case to demonstrate the presence of TCH and DHC in normal tissue from 5 patients by this method As the smallest amount of THC detectable by staining with ammonium molyb date perchloric acid reagent was about 5 γ per cm application line the maximal amount of lipid applied on the application line of the chro

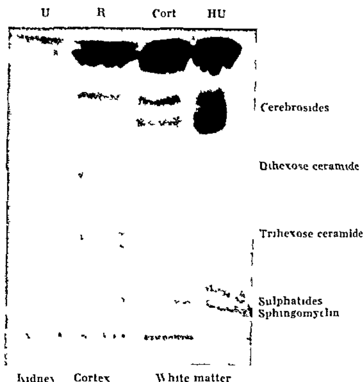


Fig 2

Thin layer chromatogram of sphingolipids isolated by saponification from kidney and brain from patient A fam II Developed and sprayed as Fig 1 The presence of trihexose ceramide in the brain is demonstrated

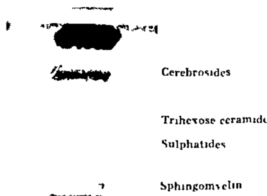


Fig 3

Globus pallidus brain pt A faml II Thin layer chromatogram of sphingolipids isolated by sapinofisation Developed and sprayed as Figs 1 and 2 The presence of trihexose ceramide is demonstrated

matogram was 1 mg per cm and the normal organs studied have a maximal total lipid content of about 20 per cent fresh weight the normal THC concentration must have been below 1 00 0 2 mg per g tissue fresh weight

Chemical characteristics of isolated glycolipids Table 1 shows the percentages of the elements in an isolated THC fraction (source of origin heart case A family II) in comparison with theoretical values As the relative oxygen content would vary greatly with the length of the carbohydrate chain the good agreement between analytical and theoretical values supports the view obtained by TLC that the isolated THC fraction is a trihexoside

THC fractions obtained from kidney A kidney B and heart B hac similar carbohydrate chains Prolonged hydrolysis yielded galactose and glucose by partial hydrolysis as described only galactose could be detected and total hydrolysis of hexose ceramide remnant with Rf ~ 0 82 (the same as the brain cerebrosides), isolated from degradation products of TCH yielded only glucose The constitution of THC was thus in all cases ceramide glucose (galactose)₃ DHC from kidney A yielded only galactose by prolonged hydrolysis indicating the constitution of DHC to be ceramide (galactose)₂

TABLE 2

Fatty Acids in THC (Source of Origin Heart Case A Family II)

(Distribution in per cent)

	%		%
$C_{16\ 0}$	2 4	$C_{21\ 0}$	1 5
$C_{18\ 0}$	7 2	$C_{22\ 0}$	23 9
$C_{18\ 1}$	0 8	$C_{23\ 0}$	2 5
$C_{19\ 0}$	trace	$C_{24\ 0}$	19 2
$C_{20\ 0}$	9 6	$C_{24\ 1}$	25 5

Table 2 shows the fatty acid composition of THC isolated from heart (case A family II) The principal fatty acids are seen to be $C_{22\ 0}$ $C_{24\ 0}$ and $C_{24\ 1}$ and the total value for unsaturated fatty acid (except $C_{24\ 1}$) is seen to be rather low This could be due to storage but the main features are the same as those obtained by *Mårtensson* (8) in normal occurring THC

Infra red spectroscopic characteristics of isolated glycolipids This analysis was carried out on the THC fractions isolated from kidney (case A family III) heart and brain (globus pallidus) (case A family II) No absorption bands for phosphate or sulphate were present The DHC fraction from kidney (case A family III) was also submitted to

infra red spectroscopic analysis. The spectrum had the same characteristics as the THC spectra.

TABLE 3

Concentration of THC in Different Organs from the Patient Material Examined

Patient	Organ (and region)	
A family I	Kidney (cortex)	10 mg per g tissue (wet weight)
A family I	Kidney (medulla)	23 " " " " "
A family II	Kidney (cortex)	8 " " " " "
A family II	Kidney (medulla)	8 " " " " " "
A family II	Heart	14 " " " " " "
A family III	Kidney biopsy	3 " " " " "
Normal kidney (calculated from *Mårtensson*'s data		0.2 mg per g tissue (wet weight)
Fabry kidney (*Sweeley & Klionsky*)		15 mg per g tissue (wet weight)

Quantitative estimation of THC. Table 3 demonstrates the results of quantitative estimation of THC in kidney cortex and medulla from *patient A, family II and patient A, family III, renal biopsy specimen* from patient A, family I and heart muscle from patient A, family II, compared with the value obtained by Sweeley and Klionsky and the normal value in kidney, calculated from Mårtensson data. To our knowledge no data have been published on the normal occurrence of THC in heart muscle. The highest concentrations were found in kidney from patient A, family III (especially in medulla), and in the heart from patient A, family II. As patient A, family III died in uraemia (serum creatinine 30.2 mg per cent), and patient A, family II died of cardiac failure, these findings are in good agreement with the clinical course of the disease. The renal function of patient A, family I and patient A, family II were much better, the serum creatinine being 2.1 mg per cent and 1.6 mg per cent respectively.

TABLE 4

THC Percentage per Weight of Total Neutral Glycolipids in Brain (Patient A, Family II)

Frontal cortex	14.4
Corona radiata	26.3
Globus Pallidus	30.0

Table 4 shows the THC percentage of total neutral glycolipids in some regions in brain from patient A, family II. It is seen that cortex is much less involved than other parts of the brain. These results are in agreement with the finding that the psychic function of patients with Fabry's disease is much less involved than motor function and the function of the autonome nervous system.

Serum and Urinary Sediment

TLC of saponified serum lipid extract from patient A and B family III demonstrated the presence of a THC fraction (Rf 0.42 in the solvent system described above). This fraction contained carbohydrate and not sialic acid or phosphorus. The Rf value showed that the carbohydrate chain was similar to the THC fraction isolated from organs. Quantitation was then performed as described above and the concentration of THC in serum from these patients were thus esctimated to 4 mg and 2 mg per 100 ml respectively (Fig. 4).

In 10 normal controls as well as in serum from the relatives of the patients A and B family I, patient A family II and patient A family III no well defined THC fraction could be demonstrated. However *Svennerholm & Svennerholm* (14) in a study on serum glycolipids were able to isolate 356 mg glycolipids from 17000 ml normal human serum and calculated the yield to be about 60 per cent based on hexose determination. 5–10 per cent consisted of THC and the carbohydrates were glucose, galactose and galactose. These figures would give a normal value for THC of 0.17 mg per 100 ml serum.

TLC-analysis of urinary sediment are further complicated by the fact that a multitude of bacterial glycolipids are present in some cases. Therefore it has not been possible to draw any conclusion from such data.

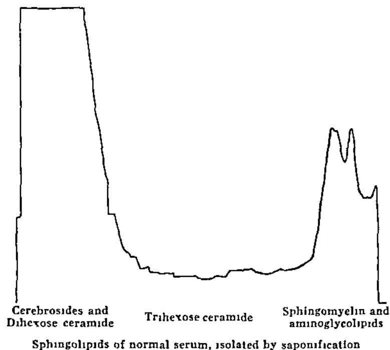
DISCUSSION

As the brain represents the most highly developed membraneous system in the human body the study of membrane lipids has to a large extent centered on this organ. The glycolipids of brain, cerebrosides, sulphatides and gangliosides have been ascribed an active and specific role in the structure and function of the brain membranes, the two former in myelin (16), the latter in cortex (7).

In the last few years however, evidence has been presented which suggests a more general role of the glycolipids in membrane function. A wide variety of glycolipids have been demonstrated in extracerebral tissue and each organ seems to have its characteristic glycolipid pattern. In erythrocytes (15) and kidney (8) the aminoglycolipids dominate, in liver (15) and spleen (15) dihexosides with different carbohydrate chains are present in highest concentrations.

The implications of this specializations to the membrane structure and function in different organs are not yet known but it is believed that the study of metabolic errors in diseases as the sphingolipidoses will shed some light on the metabolism and function of these substances.

In Gaucher's disease a deficiency of β-glucocerebrosidase has recently been demonstrated (9) with resulting accumulation of β-glucocerebroside in the organs affected. In a hypothesis presented by *Hillborg & Svennerholm* (4) a secondary release into the intercellular space takes



Sphingolipids of normal serum, isolated by saponification

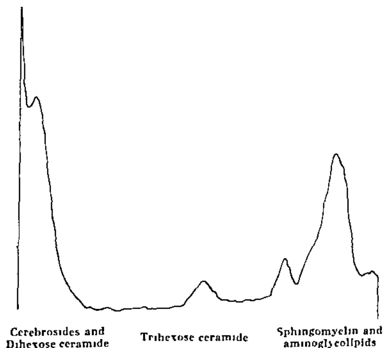


Fig 4

place, with subsequent storage in reticulum cells In accordance with this hypothesis is their finding of elevated serum cerebroside concentration in Gaucher's disease

The enzymatic defect in Fabry's disease is unknown but from the present studies it appears that in Fabry's disease a similar release mechanism may be presumed It has been shown that a dihexose ceramide, and, in particular, a trihexose ceramide with known carbohydrate content is deposited in different organs in varying concentrations The clinical course of the disease seems to a great extent to depend on the concentrations of THC in the individual organs especially in kidney, heart and brain Furthermore, the THC fraction seems to be elevated in serum from patients suffering from Fabry's disease This finding may be of value in establishing a diagnosis The sensitivity of the methods presented also opens the possibility of a biochemical diagnosis, even in cases where only minute tissue samples are available, as for example a renal biopsy

SUMMARY

Data are presented on the occurrence of trihexose ceramide and dihexose ceramide in different tissues from three patients belonging to three different families with Fabry's disease The chemical structure of the compounds is the same in the different organs studied and confirms the results of *Sweeley & Klionsky*

Furthermore a method is presented which allows determination of trihexose ceramide in serum and in small tissue samples obtained by biopsy

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RETICULOSARCOMATOSIS IN THREE SIBLINGS

By

B ZACHAU CHRISTIANSEN and H E CHRISTENSEN

Received 20 iii 66

A previous paper (*Zachau Christiansen & Rasmussen* 1963) has reported on the deaths from reticulosarcomatosis of a pair of female twins, presumably monozygotic, occurring at the age of 13 months, with an interval of 3 weeks Since a younger brother, born three years later, has now died from the same disease his case history will be reported in the following

CASE HISTORY

(BpF 1397/63)

Boy aged 10 months admitted with rise of temperature during three weeks 2 half siblings 16 and 14 years old in good health 2 twin sisters born 3 years earlier died as mentioned 13 months old of reticulosarcomatosis a brother aged 2 years is in good health

The mother was in good health during pregnancy no x ray examination nor blood transfusion normal delivery *in a maternity department birth weight 3500 g*

Development during the first 7 months normal From that time a remarkably rough tremor of arms and legs appeared though especially when he was excited 3 weeks before the admittance he had a cold with rise of temperature and cough Spontaneous decline of temperature only temporarily then again febrile though treated with penicilline at the same time tired without appetite and with frequent rather loose stools

At the admittance a rough tremor of all 4 extremities is found There is slightly increased flush and swelling of the mucosa of the throat lymph nodes swollen up to the size of a pea on both sides of the neck, in axillae and the right inguinal region

A suppurative otitis was treated with penicilline and tetracycline Later a left sided facial palsy developed A moniliasis infection of the oral cavity was treated with nystatine (Mycostatin®)

After 5 weeks transferred to the Radium Centre The boy continued to have rough tremulation in all extremities later clonic convulsions treatment with phenobarbitone and primidone (Mysoline®) without effect 2 local x ray treatments given (40 r) before death

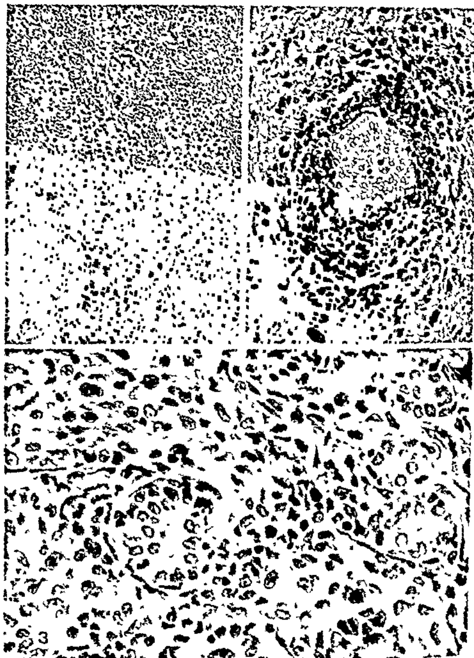
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Urine normal

Fauces No pathogenic bacteria

X ray examination Chest Slight infiltration at the left hilus
Normal urogram

Ophthalmoscopia Normal



Figs 1–3

Fig 1 *Diffuse reticulosarcomatous growth in liver* Haem.-Eos × 140

Fig 2 Cerebral perivascular infiltration of reticulum sarcoma cells intermingled with reticulin fibrils Foot staining × 240

Fig 3 *Invasive growth of the reticulum sarcoma cells to salivary gland* Haem.-Eos × 360

Biopsy from angular lymph node Sarcomatous stroma with nuclear polymorphism like reticulosarcomatosis Some gland structures assumed to be from an invaded salivary gland

Autopsy Sarcomatous tissue was found in mediastinum in columneal lymph nodes and in thymus liver (Fig 1) and pancreas and in the sublingual region (Fig 2) microscopically corresponding to the polymorph reticulosarcamatosis of Oliveira (*Oliveira* 1937) with a great number both of collagene fibrils and reticulin fibrils Bronchopneumonies were found in the lungs

Brain autopsy (Erna Christensen M D) Brain weight after formaline fixation 750 g The brain was pale with thickening of the leptomeninges Otherwise no abnormalities could be seen on the surface or at coronal sections Brain stem and cerebellum were also normal at macroscopical examination Histological examination revealed that the brain was normal according to the age In leptomeninges fibrosis was present together with slight infiltration with blood pigment containing macrophages histiocytes and lymphocytes In the brain substance perivascular cuffins of cells were present in the white substance striated bodies and brain stem Beside the mentioned cells endothelial like cells with pronounced amounts of chromatin inside the nuclei could be seen They did not contain mitoses but in between those cells a great amount of reticular fibres was present (Fig 3) which could be seen in a specimen stained for reticulin (Foot s staining) Otherwise the histological examination did not reveal any abnormalities

DISCUSSION

Our findings of reticulosarcomatosis in three siblings of which two were twins all dying about one year old seems noteworthy in view of the discussion about genetic factors entering the development of leukaemic diseases

Our first report referred to studies by others on leukaemia in identical and fraternal twins Since then *Pearson Grello & T W Cone* 1963 have reported on a pair of identical twins with leukaemia adding three further cases so that by now a total of 14 pairs are on record

Furthermore it has come to our attention that *Smith* 1961 has published the records of two brothers who died from reticulosarcomatosis at the age of 2 and 3 years respectively

As generally known there is no clear distinction between reticulosarcoma and lymphosarcoma which must be grouped together with lymphatic leukaemia and apart from myeloid leukaemia While chronic myeloid leukaemia is characterized by the presence of an unusually small chromosome in most if not in all cases (f e g *Clemmesen* 1965) no such change in the chromosome pattern is on record for lymphatic leukaemia lymphosarcoma or the related reticulosarcoma However on the background of *Smith* s observation of reticulosarcomatosis in two

brothers and of our finding of the same disease in three siblings including a pair of twins one may suggest a genetic component in the pathogenesis of also this disease The assumption of exposure to a common carcinogen some viral influence during pregnancy for example does not appear reasonable the mother being well during the pregnancies and a healthy brother born between the mentioned cases

SUMMARY

A nearly one year old boy with 2 assumed identical twin sisters who died of reticulosarcomatosis at the age of 13 months with an interval of 3 weeks developed the same disease and died It is suggested that a *genetic component may enter the pathogenesis of reticulosarcomatosis*

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EFFECT OF HEPARIN AND PLASMINOGEN INHIBITOR (EACA) IN BRIEF AND PROLONGED TREATMENT ON INTRAVENOUSLY INJECTED TUMOUR CELLS

By

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Received 2? v 66

Many authors have reported that an extremely important factor indeed a prerequisite for growth of blood borne metastases is the formation of thrombi around the tumour cells It has thus been possible to reduce the number of metastases to lungs and liver in homologous systems by treatment of the experimental animals with single doses of anticoagulants or fibrinolytics while administration of repeated heparin doses has led to a further reduction Reduction of the fibrinolytic activity of the blood has the opposite effect (cf *Boeryd* Survey of Literature 1965) *Boeryd* (1965) found however that a single dose of heparin redistributed the metastases from the lungs to the liver in an isologous system

Temporary tumour cell retention in the lungs after intravenous injection has been demonstrated in homologous systems (*Ambrus et al* 1956 *Selecki* 1959 *Griffiths & Salsbury* 1963 *Greene & Harvey* 1964) and in an isologous system (*Boeryd & Lundin* 1966) It has been found however that treatment with a single heparin dose can facilitate the transpulmonary passage of tumour cells in homologous systems (*Ambrus et al* 1956 *Koike* 1964) as well as in an isologous system (*Boeryd & Lundin* 1966)

Several workers have experimentally demonstrated a transhepatic passage of intraportally injected tumour cells (*Zeidman et al* 1956 *Jonasson* 1960 *Griffiths* 1960 *Saldeen* 1963 *Koike et al* 1963) but *Fisher & Fisher* (1961) found no increased transhepatic passage of such cells in heparinized rats But after intraportal injection of tumour cells prolonged heparin treatment for 4 or 7 days reduced the number of liver metastases but not heparin treatment for 2 days (*Fisher & Fisher* 1961)

The present investigation was designed to ascertain whether in the

This investigation was supported by research grants from the Swedish Cancer Society

same isologous system as used previously (*Boeryd* 1965) heparin and epsilon aminocaproic acid (EACA) in single doses would influence transhepatic passage and the metastasizing of intraportally injected tumour cells

Further it was designed to study the effect of prolonged heparin and EACA treatment on intravenously induced metastases The basic problem here was whether such treatment would accentuate the redistribution of metastases caused by a single heparin dose, or whether prolonged anticoagulant therapy would inhibit metastatic growth uniformly in all organs and thus confirm the importance, for the development of metastases, of a thrombus around tumour cells adhering to vascular endothelium

MATERIAL AND METHODS

The same tumour host system as in previous investigations was used (*Boeryd* 1965 *Mellgren et al* 1966) Tumour cell suspensions were prepared according to *Madden & Burk* (1961) with modifications according to *Boeryd et al* (1965) The cells were counted in a haemacytometer The mice were fed commercial pellets and water *ad libitum*

A For the investigations with intraportal injection of tumour cells the animals were devided into 3 groups Immediately before the injection of tumour cells 25 mice were injected in the jugular vein with 0 1 ml saline 54 mice with 0 1 mg heparin (Vitrum Stockholm) in 0 1 ml solution and 26 mice with 30 mg EACA (Kabi Stockholm) in 0 1 ml solution The mice were anesthetized with ether The heparin and EACA effects set in immediately and were assayed with the methods described previously (*Boeryd* 1965) The clotting time exceeded 2½ hours 45 minutes after heparin injection and 15 minutes 1½ hour after heparin injection The lysis time 45 minutes and 90 minutes after the EACA injection exceeded 4 hours and ½ hour respectively

Using a gauge 27 needle 10 000 tumour cells in 0 1 ml modified Parker 199 (*Salk et al* 1954) were injected into the main trunk of the portal vein Complications in the form of mesenteric haematoma were a serious matter being fatal in 7 controls 39 heparinized mice and 3 EACA treated mice immediately or shortly after the injection Another control mouse and 4 EACA treated mice died before the 19th day and were discarded Attempts were made to inject into one of the minor branches of the portal vein instead, but this required ligation of the vein in the heparinized mice and they later died from intestinal infarction

B For the investigations with intravenous injection of tumour cells the mice were devided into 5 groups

Group I	20 mice 15 given 0 1 ml and 5 given 0 02 ml saline subcutaneously every 8 hours for 6 days
Group II	35 mice 23 pretreated with 1 mg heparin in 0 1 ml solution and 12 pretreated with 1 mg in 0 02 ml solution subcutaneously every 8 hours for 6 days
Group III	22 mice without pretreatment
Group IV	19 mice fed a powdered diet contained 30 per cent EACA for 6 days
Group V	4 mice without pretreatment

Groups I and V were control groups for Group II and Group III a control group for Group IV

The actions of heparin and EACA were assayed with the same method as above Both the clotting time in heparinized mice and lysis time of the coagulum from EACA treated mice always exceeded 30 minutes for the entire period of treatment

50 000 tumour cells in 0 1 ml modified Parker 199 were injected into a tail vein the period of observation being 18 days for Groups I II III and IV and 12 days for Group V Before the 19th day 3 of the mice in Group I 23 of those in Group II and 2 of those in Group III died andwere discarded The deaths in Group II of all

mice except one were due to subcutaneous haematoma during the course of heparin treatment. However the mortality rate decreased when heparin was injected in 0.07 ml solution using a gauge 30 needle instead of 0.1 ml with gauge 27 needle.

In all mice the lungs and liver were examined microscopically and the number average and total volume of the metastases were calculated as described previously by *Boeryd et al* (1966). The significance of any difference between groups was evaluated with the aid of Wilcoxon's two sample rank test and Fisher's exact test (*Hyrenius* 1969). Differences with $P < 0.05$ were accepted as significant.

RESULTS

A. At autopsy of mice intraportally inoculated with tumour cells gross metastases could be found in the liver only except in one animal which had metastases in one lymph node and pelvic fat tissue.

The inoculated tumour cells yielded takes in the liver of all animals. The groups did not differ significantly in the number or average volume of metastases to the liver. The total metastasis volume tended to be higher in the heparinized mice than in the controls.

The incidence of takes in the lungs was significantly increased in the heparinized group according to Fisher's exact test (10/15 # 3/17). The number of metastases in the lung was higher in the heparinized mice but owing to the small number of positive controls statistical evaluation is unreliable. The same applies to the average metastasis volume which was smaller than that of the controls (Table 1).

TABLE 1
Metastases to Lungs and Liver after Intraportal Tumour Cell Injection

	To lungs				To liver			
	Incidence of takes	N	v 10^{-6}	N 10^{-3}	Incidence of takes	N	v 10^{-6}	N 10^{-3}
Controls	3/17	10	11	0.1	17/17	63	3740	235
Heparin	10/15	98	2	0.2	15/15	72	5331	383
EACA	5/19	27	11	0.3	19/19	47	4125	192

EACA treatment had no effect on the incidence of takes in the lungs or on the average and total metastasis volumes compared with the controls. Only the mice with metastases to the lungs gave no more information (Table 1).

B. At autopsy of mice intravenously injected with tumour cells in groups I, II, III and IV gross metastases were found usually to the liver and occasionally to the subcutis, kidneys, adrenals, mesentery, peritoneum, ovaries, lymph nodes, testes, pelvic fat tissue and skeletal muscles. There were no gross metastases in group V. Through the number of gross metastases did not differ significantly between groups the number of subcutaneous takes tended to be higher and the number of lymph node takes lower in the heparinized groups than in the relevant control group (Table 2).

TABLE 2

Gross Metastases after Intravenous Tumour Cell Injection

Group no		Total no of animals	No of animals with metastases	No of animals with metastases in Kidneys	Adrenals	Ovaries and testes	Pelvic fat	Mesentery	Peritoneum	Lymph nodes	Subcutis	Skeletal muscles
I	Controls	17	16	0	4	3	8	5	2	9	6	2
II	Heparin	12	11	2	1	6	1	2	3	2	8	0
III	Controls	20	19	7	3	11	0	9	1	8	9	2
IV	EACA	19	14	3	3	8	3	3	0	7	3	3

Heparin treatment had no effect on the incidence of takes in either lungs or liver In the lungs the number of metastases increased, the average volume tended to diminish and the total volume of metastases diminished In the liver there was no significant effect on the number of metastases but the average as well as the total metastasis volumes increased (Table 3)

EACA treatment also failed to affect the incidence of takes in lungs and liver, but the average and total metastasis volumes, in the lungs tended to increase The number of metastases to the lungs did not deviate significantly from that in the relevant control group The EACA-treated mice did not differ from the controls with respect to the liver (Table 3) These effects of heparin and EACA treatment were obtained whether all mice or just those with metastases were compared with the relevant controls

When the controls observed for 12 days (Group V) were compared with those observed for 18 days (Group III), it appeared that the number of metastases to the lungs was similar in the two groups but the average and total metastasis volumes were considerable smaller in Group V In the liver only a very few small metastases developed in 12 days (Table 3)

When Group V was compared with the heparin-treated mice in group II, it appeared that the number, average and total volumes of metastases to the lungs were similar in the two groups In the liver the same factors were considerably smaller in Group V

DISCUSSION

Mice given a single injection of heparin or EACA immediately before receiving an intraportal injection of single-cell suspension of MCGI exhibited no changes compared with control mice in the number, and average volume of metastases to the liver This bears out the

TABLE 3

Metastases to Lungs and Liver after Intravenous Tumour Cell Injection Observed after 18 Days in Groups I-IV after 12 Days in Group V

Group	All mice to lungs No of mice	N	v 10^{6}	V 10^{3}	Mice with metastases to lungs	No of mice	N	v 10^{-6}	V 10^{3}
I Controls	17	68	100	7	Controls	12	93	100	9
II Heparin	12	100	3	0 3	Heparin	7	170	3	0 5
III Controls	20	125	41	5	Controls	16	150	41	7
IV FACA	19	79	121	9	FACA	18	81	120	10
V Controls	4	111	2 3	0 3	Controls	3	146	2 3	0 3

Group	to liver No of mice	N	v 10^{6}	V 10^{3}	to liver	No of mice	N	v 10^{6}	V 10^{3}
I Controls	17	23	1005	23	Controls	15	25	1006	25
II Heparin	12	48	2337	111	Heparin	12	48	2337	111
III Controls	20	39	1800	70	Controls	13	41	1797	73
IV FACA	19	53	1216	64	FACA	19	53	1216	64
V Controls	4	4	52	0 2	Controls	3	5	52	0 3

observations of *Fisher & Fisher* (1961) in homologous rats treated with heparin for two days Accordingly any formation of thrombi around the tumour cells during the first hour after injection seems to lack significance The presence of metastases in the lungs indicates *that transhepatic tumour cell passage takes place This finding agrees* with what previous workers have reported However the higher incidence of takes in the lungs after heparin treatment suggests that anticoagulants promote transhepatic passage of tumour cells but anti fibrinolytics do not inhibit this transport In this respect the results *in heparinized animals differ from those reported by Fisher & Fisher* (1961) who found no increase of transhepatic passage of tumour cells after heparin treatment

Prolonged heparin treatment did not reduce the number of takes in either lungs or liver but increased the number of metastases to the lungs Thus the results *Clifton & Agostino* (1963 1965) reported for a homologous system were not confirmed in this system

Comparison of the liver metastases between Group II which was observed for 18 days—12 days after the end of heparin administration —and Group V which was observed for 12 days convincingly shows that these metastases evidently began to develop while heparin admini stration was in progress Hence anticoagulant therapy did not inhibit the growth of metastases in this isologous system

The number as well as the average and total volumes of the meta stases to lungs and liver in heparinized mice disclosed that prolonged treatment with an anticoagulant redistributed the metastases from the lungs to the liver This most likely indicates that the transpulmonary passage of tumour cells had been facilitated notwithstanding the fact that the number of metastases to the lungs had not been reduced The absence of a reduction in the number of metastases to the lungs sug gests that heparin treatment perhaps facilitated the continued circula tion of tumour cells in the blood (cf *Kothe* 1964), some of which on repeated passage were caught and thus able to start a metastasis Such a hypothesis is borne out by the fact that the metastases to the lungs were small and so could have been growing for a shorter time than those metastases originating from tumour cells that had been retained from the very beginning

The results of EACA treatment may be accounted for as pointed out previously (*Boeryd* 1965) by facilitated retention of the tumour cells in the lungs and perhaps also by aggregation of cells which results in larger metastases No increase in the number of extra pulmonary metastases was observed in these animals

On the basis of experimental results in homologous systems the clinical use of heparin as an agent against new metastases had been suggested by *Clifton & Agostino* (1963) and recommended by *Rift et al* (1965) The results of this and previous experiments in an isologous system disclosed however, that the heparin effect was reversed *i e*

the dissemination of tumour cells into various organs was promoted evidently owing to the facilitated transpulmonary and transhepatic passage of tumour cells

SUMMARY

Enzymatically produced tumour cell suspension of a 20 methylchol anthrene induced rhabdomyosarcoma was inoculated intraportally or intravenously in isologous CBA mice Treatment with a single heparin dose promoted the transhepatic passage of intraportally inoculated tumour cells In mice intravenously inoculated with tumour cells and treated with heparin continuously for 6 days the number of metastases increased but the total metastasis volume decreased in the lungs and the average and total metastasis volumes increased in the liver Possible reasons for these results were discussed The results obtained in untreated mice observed for 12 days compared with the results in heparin treated mice observed 12 days after the heparin treatment disclosed that anticoagulant therapy did not inhibit the growth of metastases in this isologous system

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REGENERATION OF PARIETAL PERITONEUM IN RATS

1 A Light Microscopical Study

By

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Received 23 v 66

Previous investigations on the regeneration of peritoneum have almost uniformly led to the conclusion that peritoneal defects heal very rapidly, but considerable disagreement still exists with regard to the origin of the cells forming the new mesothelial lining The opinions held by various authors on this point may be summarized as follows:

1 Peritoneal defects are covered solely by the migration of mesothelial cells from the intact peritoneum bordering on the wound (4, 5)
2 Regeneration of the mesothelial surface takes place by metaplasia of fibroblasts in the tissue subjacent to the wound, possibly also by the differentiation of monocytes and histiocytes in the wound exudate (2, 6, 10, 18, 22).
3 Mesothelial cells are detached from adjacent peritoneum and become implanted on the wound surface as free grafts, which proliferate and join together (1, 3, 12)

The proponents of the second and third of these theories have usually also found evidence that the intact mesothelium adjacent to the wound takes part in the repair process by proliferation and migration

If the first of these theories were correct, one would expect the new mesothelium to grow in from the edge of the wound at a fairly constant rate and, consequently, small wounds would heal faster than large ones

If the second theory were the right one, cells in the wound exudate (monocytes, histiocytes or fibroblasts) should gradually change their appearance to attain the characteristics of mesothelial cells, and these changes would probably occur more or less simultaneously over the whole wound surface

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Finally, if the third theory were correct one would expect to find *a*, a number of detached mesothelial cells in the peritoneal fluid, *b*, islets of mesothelial cells on the surface of fresh wounds, and *c*, a high mitotic activity in the intact mesothelium, since the postulated constant detachment of mesothelial cells must be made good by cell division

The present study was undertaken in an attempt to prove or disprove one or other of the theories mentioned In the course of this investigation however, observations were made suggesting a fourth possibility, *viz*, that new mesothelium may be formed by the differentiation of peritoneal macrophages

MATERIALS AND METHODS

A total of 67 male rats 4–6 months old and weighing 200–300 g were used 57 rats were operated on and 10 rats were used to provide samples of normal peritoneal fluid and normal parietal peritoneum In addition samples of normal peritoneal fluid were obtained from 20 rats subsequently used for experiments not included in this report In some of these animals attempts were also made to obtain mesothelial cells for smears by drawing a blunt metal spatula across the parietal peritoneum

Operative procedures The animals were operated on under anesthesia with ether alcohol (2 1) The abdomen was opened through a long midline incision and wounds were made in the parietal peritoneum in one of the following ways

1 Three burn wounds were inflicted on each side of the midline by means of a copper rod heated by rapidly circulating, thermostat-controlled hot water A detailed description of the burning apparatus has been published elsewhere (21) The flat end of the rod which had a diameter of 4 mm was applied to the peritoneum for 15 seconds with a rod temperature of 60° C, using a moderate and even pressure This procedure resulted in necrosis of the mesothelium and the subserous connective tissue whereas the underlying muscle survived

2 Mechanical, circular wounds were made by rotatory movements with a cork borer followed by stripping of the tissue within the circle by means of anatomical forceps The serous membrane and at least one layer of the abdominal muscle were removed *en bloc* On each side of the midline were made either *a* three wounds with diameters 4 mm or *b* two wounds with diameters 12 mm or *c* one wound with a diameter of 36 mm

The small and the medium sized wounds were placed at least 10 mm apart and in areas devoid of major blood vessels Arteries and veins crossing the large wounds were cauterized just peripheral to the wound edge

The abdominal incision was closed in two layers with silk sutures

The rats were re-opened under anaesthesia after 1, 4 12 or 24 hours 2 3 4 6 8 10 or 14 days The midline incision was opened by cautery to minimize bleeding Peritoneal fluid samples were obtained by a capillary pipette for smears and by a graded pipette for cell counts 10 cmm of peritoneal fluid was mixed with 990 cmm of 0 05 per cent methyl violet and nucleated cells counted in a Fuchs R senthal chamber

Histological Procedures

Smears of peritoneal fluid were fixed in equal parts of 96 per cent alcohol and ether for 1 2 hours and stained according to Papanicolaou Differential counts were made by counting 200 nucleated cells in each preparation

Sections The peritoneal membrane with the wounds on one side of the midline was fixed in 1 per cent osmium tetroxide in 0 1 M phosphate buffer at pH 7.3 for 1 2 hours Alternatively fixation was carried out in 2 5 per cent glutaraldehyde in 0 1 M phosphate buffer for 2 hours followed by osmium tetroxide solution for 1 hour Fixation was started in the living animal by dripping ice-cold fixative on to the wounds and the adjacent peritoneum for 1 minute Thin slices of tissue were then cut out and immersed in the fixative Small pieces were cut out from

the central and peripheral parts of the large wounds whereas the small and medium sized wounds were fixed *in toto* along with a brim of the surrounding peritoneum The specimens were kept at 4° C during fixation

When fixation was complete the specimens were trimmed dehydrated in graded acetones and embedded in Vestopal W They were oriented in the blocks so that sections could be cut vertical to the peritoneal surface Sections were cut about 1 μ thick on a Huxley microtome They were transferred to glass slides and stained with 0 1 per cent toluidine blue for 30–40 seconds at approximately 80° C

Hautchen preparations The peritoneal membrane with the wounds on the other side of the midline were used for Hautchen preparations which were made with slight modifications of the method described by *Efskind* (5) The skin was dissected off the abdomen and the abdominal wall was cut out briefly rinsed in 5 per cent glucose solution and pinned out flat on a plastic disc by means of stainless steel pins It was then immersed in 0 25 per cent silver nitrate solution for 30 seconds and after a second rinse in glucose fixation took place in buffered neutral formalin for 6–8 hours In some cases the silver nitrate treatment and the second rinse in glucose was omitted

The specimens were dehydrated in alcohol as follows 50 per cent alcohol for 30 minutes 60 per cent for 1 hour, 70 per cent for 10–14 hours 80 per cent for 4 hours 96 per cent for 4 hours and absolute alcohol for 12 18 hours the absolute alcohol was changed once Finally the specimens were immersed in ether alcohol for 1 hour When removed from this mixture they were placed with the peritoneal surface upwards and horizontal As soon as the last drop of ether alcohol had evaporated but while the tissue was still moist the peritoneal surface was covered with a rather thick layer of collodion[1] When the collodion had hardened the specimens were left to soak in 70 and 50 per cent alcohol for a total of 30–60 minutes The collodion could then be stripped off as a thin film to which the superficial cell layer adhered

The collodion films were stained with hematoxylin or hematoxylin and eosin dehydrated in alcohol cleared and mounted as whole mounts To prevent later gas bubble formation in the mounting medium the preparations were placed in an exsiccator for the first few minutes until most of the solvent had evaporated

RESULTS

The observations made in the present study on the cytology of normal parietal peritoneum were fully conforming with the description and illustrations published by *Efskind* (5) A new description of this tissue was therefore considered superfluous

A *Peritoneal Fluid Cells*

Cell counts from the peritoneal fluid of 20 normal rats gave values ranging from 63 000 to 244 000 nucleated cells cmm, with an average value of 139,000 cells cmm

Differential counts of peritoneal fluid cells in smears from 20 normal rats gave these results (Table 1)

TABLE 1

Per Cent Distribution of Cell Types in Differential Counts of Normal Peritoneal Fluid Smears (20 Rats)

	Lymphocytes	Macrophages	Eosinophils	Mast cells
Range	30 70	30 56	1 29	0 5–6
Average	51	40	6 5	2 5

[1] Malinckrodt Chemical Works New York N Y

Polymorphonuclear granulocytes were not observed in normal peritoneal fluid

These results are in good correspondence with the results published by others (11 15 16)

In operated animals the peritoneal fluid contained the same cell types with a varying admixture of polymorphonuclear granulocytes which were especially numerous during the first three days after the operation Cells which could be identified as mesothelial cells were not observed in either normal or operated animals

B *Macroscopical Observations of Peritoneal Wounds*

Adhesions to the wounds were found in only 4 animals in each case a part of the omentum or the peritesticular fat was adherent to the edge of one of the large wounds

During the first two days the wounds appeared as punched out defects in the abdominal wall with an uneven hemorrhagic surface The difference in level between the wound surface and the surrounding peritoneum progressively decreased The surface of the wound was still red and velvety at 3 and 4 days and then became gradually more pale smooth and shiny At 8 days the whole wound surface was smooth and glistening though still slightly more red than the surrounding peritoneum At 14 days only a shallow depression of greyish white colour indicated the location of the wound

C *Microscopical Observations*

One hour after the wounds were made fibrin strands were seen forming a network over the wound and scattered cells were entangled in its superficial parts (Figs 1 and 2) At 4 hours the number of cells had increased considerably (Fig 4) The majority of these cells were of the same appearance as the peritoneal macrophages (compare Figs 2 and 3) with occasional mast cells in between A remarkable feature at these early stages was the contrast between the number of cells seen on the surface and the complete lack of cellular infiltration in the depth of the wound (Figs 1 and 4)

Figs 1 4

Fig 1 Section from the central part of a wound at 1 hour Macrophages and a mast cell (MC) trapped between fibrin strands (F) NM = necrotic muscle × 800

Fig 2 Hautchen preparation from a wound at 1 hour The majority of cells found on the wound surface are of macrophage appearance × 800

Fig 3 Smear preparation of normal peritoneal fluid cells for comparison with Fig 2 Macrophages (M) lymphocytes (L) and an eosinophil granulocyte (E) × 800

Fig 4 Section from the central part of a wound at 4 hours Numerous macrophages and a few mast cells between fibrin strands in the superficial part of the wound NM = necrotic muscle × 800


MC
F
NM
1


L
M
E
2
3


NM
NM
4

During the next few days the number of cells steadily increased In sections of wounds at 24 and 48 hours the cells resting on the superficial fibrin layer might resemble mesothelial cells due to their flattened form (Fig 5) When seen *en face*, however the majority of surface cells were of macrophage appearance (Figs 6 and 7) A few cells had lost their distinct cell outline and both the nucleus and cytoplasm stained less deeply presumably because the cells had flattened out and become thinner When the wounds were treated with silver nitrate before fixation the exudate between the surface cells stained brownish black (Fig 8)

At 3 days a considerable amount of fibrin was still present in the wound A large number of closely packed macrophages were seen in the superficial parts often contrasting sharply with the relatively small number of cells in the deeper parts (Fig 9) In Hautchen preparations the majority of cells had pale staining oval or reniform nuclei with more conspicuous chromatin a pale cytoplasm and indistinct cell borders (Fig 10) The intercellular spaces as visualized by silver nitrate treatment appeared broad and irregular (Fig 11)

At 4 days little or no fibrin was demonstrable in the wound exudate and large numbers of cells were present evenly distributed throughout the wound (Figs 12 and 15) When seen *en face*, most of the surface cells were large with round or oval pale nuclei 2 or 3 conspicuous nucleoli were usually present but their number varied between 1 and 5 The silver lines between the cells were incomplete and of irregular width However considerable variations in the silver line pattern were observed from one area to another in the same preparation as will be seen by comparing Figs 13 14 with Figs 16 17

At 6 days the silver lines were more complete discrete and uniform Occasional gaps between neighbouring cells were still observed both in Hautchen preparations and in sections (Figs 18 20) The cells varied markedly in size and shape

At 8 days the cells on the surface of the wound invariably formed a continuous layer without demonstrable gaps between the cells (Figs

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Figs 5 8

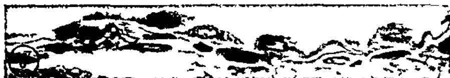
Central part of peritoneal wounds at 24 and 48 hours

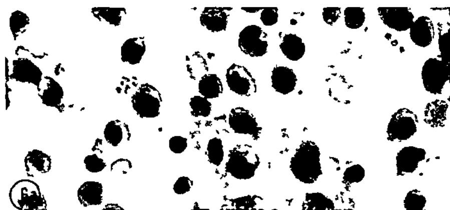
Fig 5 Section 24 hours Flattened macrophages faintly resembling mesothelial cells creeping on fibrin strands × 800

Fig 6 Hautchen preparation 24 hours A few cells stain less intensely and show a less distinct cell outline than the typical macrophages × 900

Fig 7 Hautchen preparation 48 hours A large proportion of the cells have pale staining nuclei and cytoplasm and the cell outline is often hardly visible × 960

Fig 8 Hautchen preparation 48 hours The exudate between the cells has been stained by silver nitrate treatment × 960









21–22) Häutchen preparations revealed discrete and uniform silver lines The size and shape of the surface cells were still varying, the nucleoli were smaller and of a more regular shape than at the earlier stages (Figs 23 and 24) Although the picture was not yet identical with that of normal mesothelium, there could be no doubt that both the small and the large wounds had healed, in the sense that they were completely covered with mesothelial cells

At 10 days the surface cells were flatter, the size and shape of the cells showed less variation and, accordingly, the mosaic pattern formed by the silver lines was more regular (Figs 25 and 26)

At 14 days, the picture presented by the surface cells could hardly be distinguished from that of normal mesothelium (Figs 27–30).

The changes described above are considered to be representative of the "normal" sequence of events observed in the surface of healing wounds, and they occurred simultaneously over the whole wound surface However, at the various stages there were considerable variations in the size and shape of the cells, in the nuclear size and in the intercellular relationships (Figs 31–37) Cells with two or more nuclei, which might differ in size, were seen (Figs 33 and 34)

The intact mesothelium surrounding the wound reacted to the injury largely as described by *Efskind* (5) From the second day onwards, the intercellular lines were broader than normal, and in sections the cells assumed a round or cuboidal shape These changes were interpreted to indicate a migratory activity The difference between the intact mesothelial cells peripheral to the wound and the cells on the surface of the wound exudate was clear-cut during the first 3 days after the injury Later, a distinction between the two cell populations became increasingly difficult or impossible, since the cells on the wound surface rapidly became more like mesothelial cells, and the latter assumed a less uniform appearance during proliferation and migration

There was no reason to doubt that the mesothelium peripheral to the wound played an active part in the regeneration An indication of this was found in the lively mitotic activity observed in the mesothelium immediately peripheral to the wound Quite a few mitoses were observed on the second day, and their number was maximal on the fourth day (Fig 38) The mitotic activity was limited to a zone 1–3 mm broad

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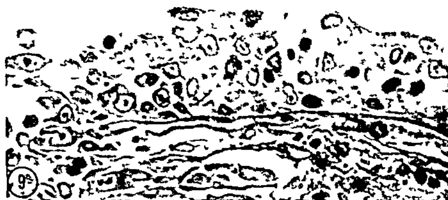
Figs 9 11

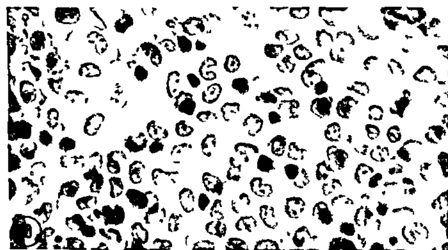
Central part of wound at 3 days × 800

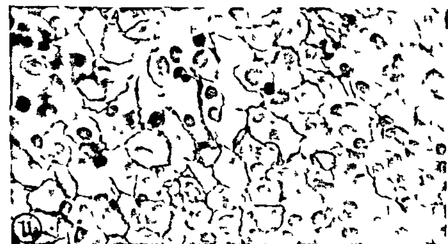
Fig 9 Section Conspicuous nucleoli are present in most nuclei Note the contrast between the closely packed cells on the surface and the relatively small number of cells in the deeper part of the wound

Fig 10 Hautchen preparation from an area in which the silver nitrate has failed to bring forth the outlines of most cells

Fig 11 From another area of the same preparation focused on the silver lines







around the wound Outside this belt, the number of dividing cells rapidly decreased, and at some distance from the wound, few or no cells were observed to be in mitosis at any stage

Dividing cells were frequently concentrated to certain areas, whereas neighbouring areas at the same distance from the wound showed very few or no mitoses

A high mitotic activity was also found in the surface cells within the wound However, in these cells the mitotic activity appeared to lag behind by approximately 24 hours Cells in division were occasionally seen at 3 days, and their number reached maximum at 4 and 6 days (Figs 39 and 41) At 8 days, a considerable number of cells were seen in division within the limits of the wound, while mitotic activity in the mesothelium outside the wound had almost ceased

DISCUSSION

Much of the discrepancy between the conclusions reached in previous investigations on peritoneal regeneration no doubt arises from the difficulty of identifying mesothelial cells in histological sections *Efskind* (5) stressed the necessity of using Häutchen preparations which permit the inspection of the surface cells *en face*, whereas most previous and later authors disregarded this problem

The same difficulty has been encountered in a related field of research, *viz*, that concerned with the regeneration of vascular endothelium Here, the conclusions have been reached that endothelial cells seen in sections by light microscopy cannot be identified with certainty (9), that *en face* preparations are essential for identifying endothelial cells (14 20), and that there are no reliable criteria for identifying isolated endothelial cells (17)

The experience gained in the present study fully confirms that these statements are valid for mesothelium as well This is not surprising since endothelium and mesothelium are morphologically so similar that they can hardly be distinguished from each other by light microscopical examination

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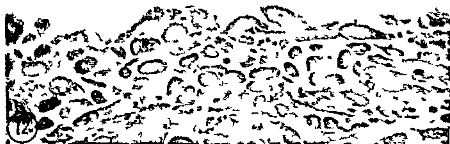
Figs 12 14

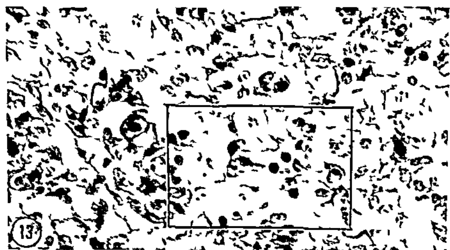
Central part of wound at 4 days

Fig 12 Section no remarkable difference from the picture at 3 days (see Fig 9) × 800

Fig 13 Hautchen preparation The surface cells are large and have pale nuclei The silver lines between the cells are incomplete and of uneven width × 320

Fig 14 Higher magnification of the area outlined in Fig 13 showing the large and dense nucleoli of the surface cells and the broad silver lines The dark nuclei out of focus belong to cells underneath the surface layer × 800


12


13


14

In the early stages of peritoneal repair, sections frequently reveal surface cells which, due to their flattened form and alignment parallel with the surface, may closely resemble mesothelial cells (see Figs 5 and 9) When the cells at these stages are examined *en face*, however, it is evident that they lack the criteria of mesothelial cells (Figs 6, 7, and 10) The large variability in the size and shape of the surface cells, the intercellular relationships, the number and size of nuclei and nucleoli and the incidence of mitoses cannot be satisfactorily established by the examination of sections alone

Hautchen preparations also have certain drawbacks First, the methods for preparing Hautchen are very time-consuming, since each preparation must be processed individually Secondly, the surface cells embedded in the collodion film are not lying in a single plane like the cells seen in a section Although this does not materially reduce their value for microscopical study, optimal microphotographs are frequently difficult to obtain Thirdly, in fresh wounds several cell layers often adhere to the collodion, therefore a picture of the surface cells is to some degree blurred by the presence of underlying cells This happens particularly during the first 48 hours

In an effort to overcome the limitations of ordinary sections, *Ellis et al* (6) also studied smears of the wound exudate, and claimed to have observed mesothelial cells among the other cells in the wound exudate as early as 24 and 48 hours after the wounds had been made However, judging from the published illustration of a smear at 48 hours, one is not easily convinced that any of these cells can be identified as mesothelial cells

Cameron et al (3) observed patches of elongated lining cells "very like those found on the normal surface, but sometimes with larger cell bodies", in sections from wounds of the liver capsule Some of these lining cells were observed as early as 2 and 6 hours after the operation At 48 hours the surface was partly covered, and at 3 days the surface of the wound was "almost completely roofed by fairly plump, low cuboidal or elongated lining cells' In support of their highly interesting graft theory the authors referred to the following experiments

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Figs 15 17

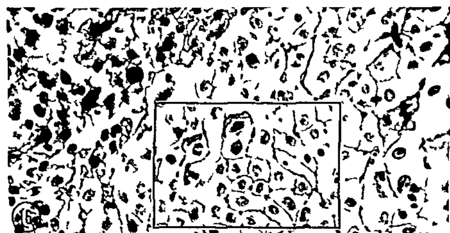
Another series of illustrations from the central part of a wound at 4 days

Fig 15 Section The surface cells in this area are large, and the cells to the right appear to be closely associated × 800

Fig 16 This micrograph is taken from the same preparation as that depicted in Fig 13 but from an area in which the silver lines form a more regular pattern × 320

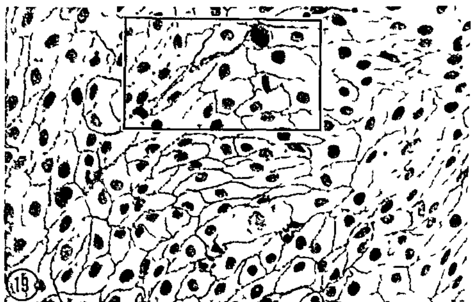
Fig 17 Higher magnification of the area outlined in Fig 16 The silver lines are still incomplete and of highly varying width Several cells are in mitosis (arrows) × 800

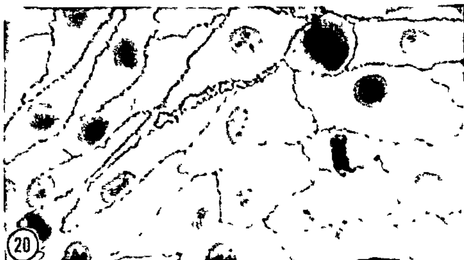

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16


17


18


19


20

1 If a glass spatula was drawn gently over the peritoneum 1–3 times and a smear was made, large peritoneal cells were seen among the other cells in the smear

2 If a coverslip was inserted between the liver and the parietal peritoneum, "lining cells are seen to detach themselves and settle on the coverslip". Very little fluid accompanied the cells so that it was unlikely that they were derived from the free peritoneal exudate The authors apparently disregarded the fact that the peritoneal fluid of rats contain, on the average, more than 100 000 cells cmm, and that nearly half of these cells are large macrophages which will readily adhere to a glass surface

Cameron et al further postulated that the peritoneal membrane "appears to be in a state of flux whereby showers of cells are constantly being detached and replaced" Various circumstances contradict this hypothesis In the first place, if this theory were correct, one would expect to find a high proportion of detached mesothelial cells in the peritoneal fluid This has not been reported by authors who have studied the composition of the peritoneal cell population (11, 15, 16, 19) In the present study, cells which could be identified as mesothelial cells were not observed in smears of peritoneal fluid from normal or

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Figs 18–20

Central part of wound at 6 days

Fig 18 The surface cells are large and flat but do not yet form a continuous layer Note the large nucleoli × 800

Figs 19 20 Hautchen preparations The silver lines are thinner and more uniform than at the earlier stages but occasional gaps between neighbouring cells are seen Fig 19 × 320, Fig 20 × 800

Figs 21–26 See page 370

Figs 21 22 Sections from different areas in the central part of the same wound at 8 days The surface cells form a continuous layer × 900

Figs 23 24 Hautchen preparations, 8 days The silver lines are discrete and uniform Nucleoli are less conspicuous than at the earlier stages Mitotic activity is still considerable Fig 23 × 320, Fig 24 × 800

Figs 25–26

Central part of wound at 10 days

Fig 25 Section A continuous layer of large flat cells cover the surface × 900

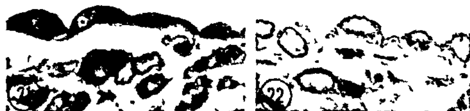
Fig 26 Häutchen preparation The cells are of a more uniform size and shape and mitoses are less frequent than at the earlier stages × 320

Figs 27 30 See page 371

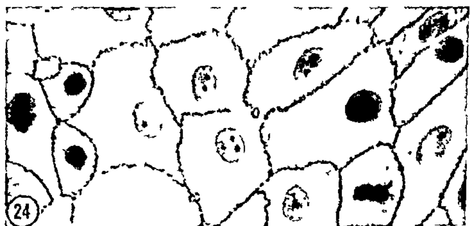
Central part of wound at 14 days

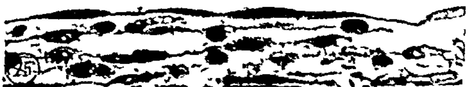
Figs 27–28 Sections Flat cells of uniform thickness form a continuous surface layer Cytoplasmic extensions from adjoining cells sometimes enclose spaces resembling large vacuoles Fig 27 × 800 Fig 28 × 900

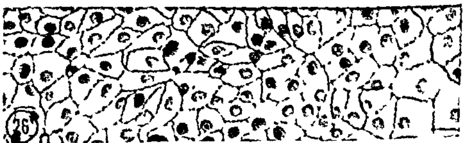
Figs 29 30 Häutchen preparations The surface cells can hardly be distinguished from those of normal mesothelium Fig 29 × 320 Fig 30 × 800







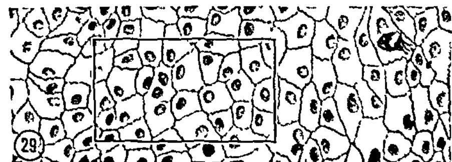


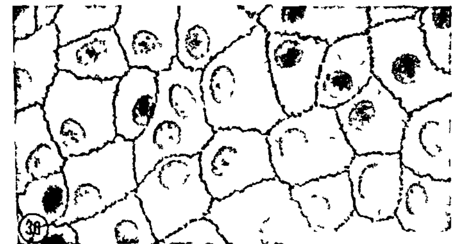


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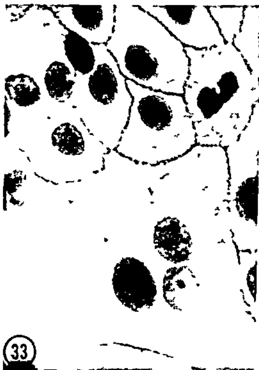


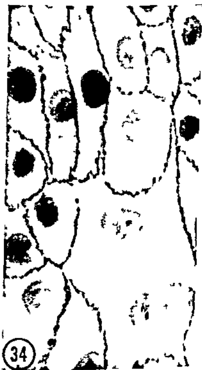


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operated rats *Efskind* (5) stated that cast off mesothelial cells were rapidly destroyed and had no further possibilities of development *Felix & Dalton* (8) found that 0 5 to 2 per cent of peritoneal fluid cells in mice were mesothelial cells but these cells were either dead or dying

Secondly if the peritoneum were in a state of flux one would expect to find a high mitotic rate in normal mesothelium since the postulated constant loss of mesothelial cells must be made good by cell division This is certainly not the case Mesothelium is generally classified as a non renewing tissue (13) *Efskind* (5) found cell divisions only once in normal rabbit mesothelium and in the present study mitoses were not observed at all in the parietal peritoneum of normal rats

In operated animals on the other hand a lively mitotic activity is evident in the mesothelium but this activity is limited to a narrow zone bordering on the wound

Hertzler (10) stated that considerable force must be used to remove mesothelial cells for microscopy Even when a needle was drawn forcefully across the peritoneum the cells tear but do not loosen In the present study attempts were made to obtain smears of mesothelial cells by drawing a blunt spatula across the parietal peritoneum These attempts were unsuccessful unless a scraping force was used in that case small sheets of mesothelial cells which were obviously damaged could be seen

All of these observations speak against the concept that the peritoneal membrane is in a state of flux

Of the previous investigators studying peritoneal regeneration only *Efskind* systematically used Hautchen preparations in addition to or

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Figs 31 34

Hautchen preparations showing cytologic variations in the surface cells of regenerating wounds

Fig 31 Mitoses in a very large surface cell at 4 days × 800

Fig 32 The long diameter of the large nucleus measures approximately 25 μ 6 days × 900

Figs 33–34 Large cells with two or more nuclei occasionally of different size are sometimes observed Fig 33 at 6 days × 900 Fig 34 at 10 days × 800

Figs 35–37 *See page 374*

Hautchen preparations showing variations in the size of cells and nuclei at 6 days. The diameters of the cell in Fig 36 measure approximately 200 and 130 μ Fig 35 × 600 Fig 36 × 480 Fig 37 × 800

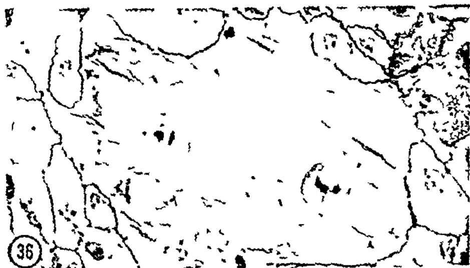
Figs 38 41 *See page 375*

Hautchen preparations illustrating the mitotic activity in and around regenerating wounds

Fig 38 Mesothelium immediately peripheral to the wound at 4 days × 370

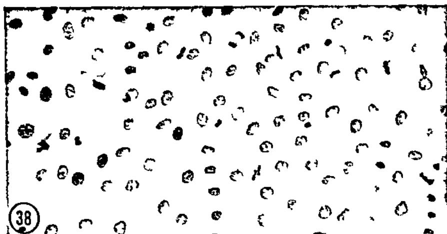
Figs 39 41 Mitoses in the central part of the wound Fig 39 at 4 days × 800 Fig 40 at 6 days × 900 Fig 41 at 4 days × 900

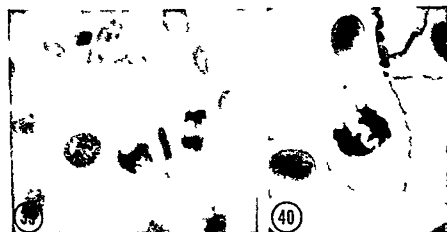






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dinary sections He concluded that regeneration of mesothelium took place by the migration of mesothelial cells from adjacent peritoneum, and that neither fibroblasts nor histiocytes took part in the regeneration of mesothelial defects

The wounds studied by *Efskind* were small burns, measuring at the most 2 mm across It is highly probable that the proliferative capacity of the intact mesothelium is so high that small defects are covered solely or chiefly from this source Besides, even if new mesothelium were also formed from cells in the wound exudate, it would be very difficult to distinguish between mesothelial cells from different sources in wounds of this size

In the present investigation, wounds which measured 36 mm in diameter were completely covered with new mesothelium within 8 days This amazing speed of healing cannot be explained by the migration of mesothelial cells from the edges, unless one assumes that mesothelial cells are capable of migrating many times faster than any other cells This holds true even if it is postulated that migration starts the moment the wound is made, which it certainly does not

The peritoneal fluid in rats contains on the average 50,000 60,000 macrophages cmm It is generally agreed that cells of this kind will readily stick to a fibrinous surface Knowing that a cell suspension of this density is constantly bathing the surface of any peritoneal wound, it is not surprising that large numbers of macrophages are found in the fibrin scaffolding before any inflammatory cells are found in the depth of the wound

Ellis et al (6) observed that the cells on the surface of peritoneal wounds were frequently separated from the cells in the deeper parts by several layers of fibrin, but did not comment upon the origin of the surface cells In my opinion, there can be no doubt that the cells observed on the surface in the early stages are derived from the peritoneal fluid If they had migrated from the blood, one would expect the polymorphonuclear granulocytes to be the first cells to arrive Instead, with the exception of some mast cells, which are also normal inhabitants of the peritoneal fluid, the cells present in the early stages are exclusively macrophages The identity of these cells has been verified by electron microscopy (7)

Later, cells from the blood and tissues (monocytes and histiocytes) invade the wound, the same way as they invade wounds elsewhere in the body However, up to the third day, a distinction can frequently be made between the surface of the wound, with a closely packed population of macrophages, and the deeper part, where a relatively small number of scattered cells are observed (see Fig 9) After the third day the fibrin disappears and the whole wound is filled with macrophages and fibroblast like cells, which are evenly distributed and give no clue as to their origin (Fig 12)

There is no evidence that the macrophages first observed in the

wound are later cast off or die On the contrary, the observations indicate that they remain viable, gradually changing their appearance to be more like mesothelial cells

The observations made in the present study are interpreted in the following way Defects in the peritoneum are rapidly covered by macrophages, which are present in large numbers in the peritoneal fluid bathing the wound surface, and therefore immediately available Later the wound is also invaded by similar cells (monocytes and histiocytes) from the blood and tissues Cells from either source differentiate to fibroblasts, and the superficially located cells gradually attain the characteristics of mesothelial cells

At the same time, the intact mesothelium surrounding the wound takes part in the repair process by proliferation and migration, the same way as epithelial cells do elsewhere in the body Small defects are probably covered chiefly by the proliferation of adjacent mesothelial cells, whereas the major part of the new mesothelium covering large wounds is derived by metaplasia of undifferentiated cells in the wound exudate The peritoneal macrophages represent a major source of these cells

SUMMARY

Peritoneal regeneration was studied in adult male rats Burns and excision wounds of varying size were made in the parietal peritoneum and studied in sections and Häutchen preparations at intervals ranging from 1 hour to 14 days The small and the large wounds healed in the same time and with amazing rapidity Within 8 days the wound surface was completely covered with new mesothelium The observations indicate that, in the large wounds, the major part of the new mesothelial cells were derived by metaplasia of undifferentiated cells in the wound exudate, and that the peritoneal macrophages represent a major source of these cells The intact mesothelium surrounding the wound contributed to the repair by proliferation and migration, and in very small wounds this source of new mesothelial cells is probably the most important

The observations made in the present study do not support the hypothesis that mesothelial cells are detached from nearby peritoneum and become implanted in the wound as free grafts

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REGENERATION OF PARIETAL PERITONEUM IN RATS

2 *An Electron Microscopical Study*

By

GUNNAR ESKELAND and ASMUND KJÆRHEIM

Received 23 v 66

It is generally accepted that peritoneal defects are rapidly covered with mesothelium, but opinions differ with respect to the origin of the new lining cells In a previous investigation the regeneration of parietal peritoneum in rats was studied by light microscopy of sections and of Häutchen preparations (5) The observations led to the tentative conclusion that macrophages deposited from the peritonal fluid may contribute to the formation of new mesothelium covering serosal defects

The present study was undertaken in the expectation that the electron microscope would provide the means for a definite identification of the cells taking part in peritoneal regeneration The ultrastructural changes which occur during the healing of peritoneal wounds have not, to our knowledge, been studied previously

MATERIALS AND METHODS

The materials and methods used in this study were essentially the same as those employed previously (5)

Thermal wounds with diameters 4 mm and deep excision wounds with diameters 4 mm 12 mm and 36 mm were made in the parietal peritoneum of adult male rats After periods of time ranging from 1 hour to 14 days the rats were re opened under anaesthesia using cautery to minimize bleeding

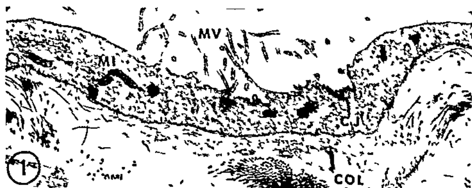
Peritoneal fluid was collected by means of a capillary pipette and immediately blown into a centrifuge glass containing ice-cold fixative The resulting cell suspension was centrifuged at 1 100 G for 10 minutes and fixation was continued at 4° C

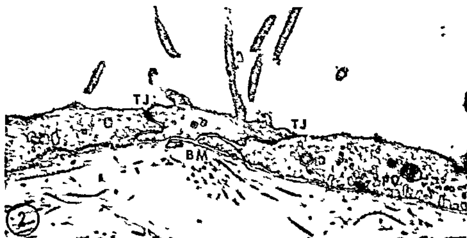
Fixation of the wounds with the surrounding peritoneum was initiated in the living animal by dripping ice cold fixing solution on to the peritoneal surface for 1 minute Thin slices of the abdominal wall were then cut out and immersed in the fixative Small pieces were excised from the central and peripheral parts of the large wounds while the small and medium sized wounds together with a brim of the adjacent peritoneum were removed in one piece

Normal peritoneal fluid and specimens of normal peritoneum were obtained from

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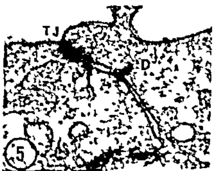
This investigation was supported by grants from Doktor Alexander Malthes Legat from *The Norwegian Cancer Society* (Landsforeningen mot kreft) and from *The Norwegian Research Council for Science and the Humanities*


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non operated rats and were treated in the same way as corresponding samples from the operated animals

Fixation was performed either in 1 per cent osmium tetroxide in 0 1 M phosphate buffer for 1–2 hours or in 2 5 per cent glutaraldehyde in 0 1 M phosphate buffer for 2 hours followed by osmium tetroxide for 1 hour The pH of the fixing solutions was 7 3 and the specimens were kept at 4° C for the remainder of the fixation period They were then trimmed dehydrated in graded acetones and embedded in Vestopal W The specimens from wounds and from normal peritoneum were oriented in the blocks to permit sectioning vertical to the peritoneal surface

Thin sections were cut on a Huxley microtome from the central part of the small wounds and from the central and peripheral parts of the large wounds They were stained with lead citrate (16), some of them also with 1 per cent uranyl acetate in 30 per cent methanol The sections were then examined in a Siemens Elmiskop I fitted with a double condenser and a 50 μ objective aperture

RESULTS

The observations made on the ultrastructure of peritoneal macrophages correspond well with those previously reported (3, 7, 13) Normal peritoneal fluid contains macrophages, lymphocytes, eosinophil cells and mast cells Peritoneal fluid from operated rats contained, in addition, some polymorphonuclear granulocytes, which were chiefly observed during the first three days following the operation No cells which could be identified as mesothelial cells were observed in the peritoneal fluid of either normal or operated animals

The ultrastructure of peritoneal mesothelium has been studied in mice (8), rabbits (1), and rats (9, 14) In the present investigation, normal parietal peritoneum was examined with the primary purpose of establishing criteria to distinguish mesothelial cells from macrophages and connective tissue cells in general Illustrations of normal peri

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Figs 1–5

Normal rat parietal peritoneum

Fig 1 Parts of two mesothelial cells showing a cell junction (CJ), characteristic microvilli (MV), and rod shaped mitochondria (MI) Collagen fibrils (COL) run in different directions underneath the mesothelium × 12 000

Fig 2 Junctions between three mesothelial cells Tight junctions (TJ) are present between adjacent cells close to the free surface The basement membrane (BM) and numerous pinocytotic vesicles (PV) are visible × 24 000

Fig 3 Occasionally a single cilium (CI) is observed projecting from a mesothelial cell × 24 000

Figs 4 5 *Tight junctions (TJ) are regularly observed between adjacent mesothelial* cells whereas desmosomes (D) are rare in normal mesothelium × 60 000

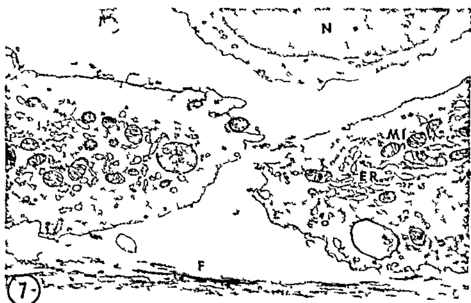
Figs 6–8 See page 382

[illegible] wound [illegible] a FR [illegible] 2 000

Figs 9 10 See page 383

Wounds at 24 and 48 hours Macrophages in in[illegible] numbers and gradually more closely packed cover the wound surfa[illegible] fibrin ML = muscle
Fig 9 × 5 000 Fig 10 [illegible] 6 000



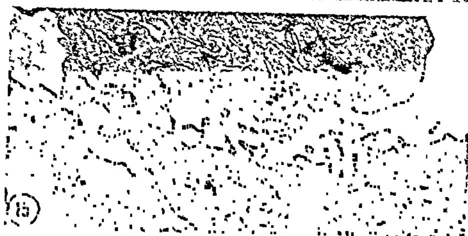




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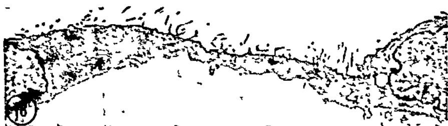


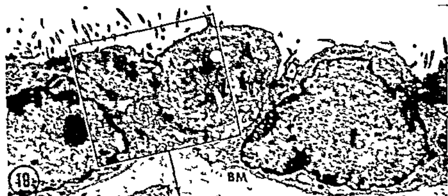






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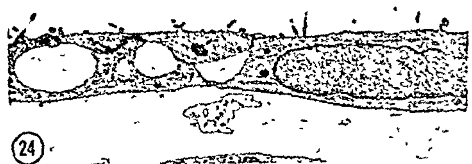
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Observations of Peritoneal Wounds

As early as 1 and 4 hours after the wounds had been made numerous cells were seen scattered or in small aggregations entangled in a network of fibrin strands (Figs 6–8) With the exception of occasional mast cells (not illustrated), all the cells at these stages were macrophages identical in appearance with the peritoneal macrophages and present in the superficial parts of the wound only

During the next 3 days the number of cells in the wound steadily increased and the cells became more closely packed filling all parts of the wound (Figs 9 11) Occasional polymorphonuclears were seen, but the vast majority of the cells were of macrophage appearance The only features which to some degree distinguished these cells from normal peritoneal macrophages were their flatter form and a gradually increasing amount of endoplasmic reticulum

At 4 days a large proportion of the surface cells were elongated and flat and some of them exhibited long cytoplasmic extensions parallel with the wound surface (Fig 12) The most conspicuous feature was an abundant endoplasmic reticulum the membranes of which were stud

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Figs 21 24

Figs 21 22 At 10 days the surface cells are usually flat and exhibit long microvilli and an abundance of rough surfaced endoplasmic reticulum (ER) Tight junctions (TJ) are regularly present Fig 21 × 6 000 Fig 22 × 24 000

Fig 23 At 14 days the endoplasmic reticulum is still conspicuous and the number of pinocytotic vesicles relatively small Except for these features the surface cells at this stage can hardly be distinguished from normal mesothelial cells × 12 000

Fig 24 The cytoplasm of two or more surface cells sometimes meet in such a way that spaces are created between the cells Identical spaces have been observed in newly formed endothelium (15) In the light microscope such spaces may appear to be cytoplasmic vacuoles × 12 000

Figs 25 29 See page 390

Junctional complexes are regularly observed between the surface cells from the 8th



Figs 30 34 See page 391

Fig 30 Generally only one desmosome (D) is seen between adjacent cells occasionally two are present along a cell junction Fine filaments (arrows) extend for a considerable distance into the cytoplasm from the desmosome plates × 40 000

Fig 31 A single cilium (Cl) is occasionally observed in a surface cell at 14 days × 12 000

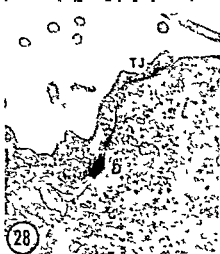
Fig 32 Bundles of fine filaments (arrow) present in the cytoplasm of surface cells at 10 days × 24 000

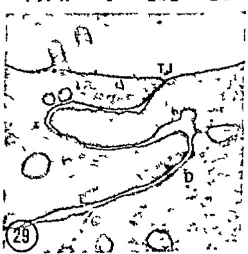
Figs 33 34 Fine filaments (arrows) are demonstrable in the microvilli of surface cells at 10 and 14 days × 60 000



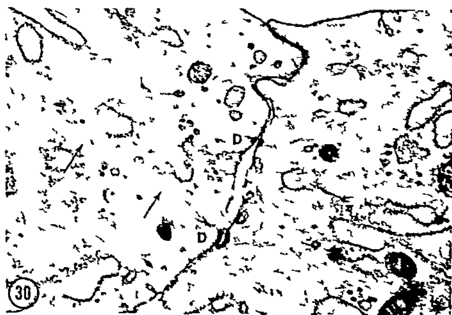


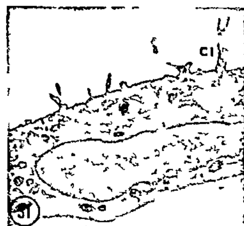




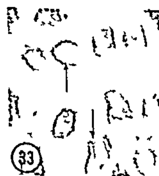


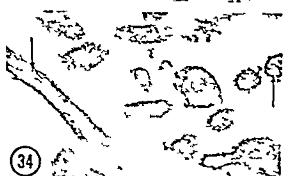
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ded with ribosomes Numerous free ribosomes were also present in the cytoplasm Large nucleoli were seen in most nuclei

At 6 days, the rough-surfaced endoplasmic reticulum was even more conspicuous (Figs 14 and 15) The cytoplasmic processes on the free surface of the cells were numerous and of a more uniform appearance The cells frequently contained lipid in cytoplasmic vacuoles The intercellular spaces were now extremely narrow, at least along some part of the borderline between adjacent cells Usually, no typical junctional complex was observed, but in some places a short obliteration of the intercellular space was seen together with a condensation of the adjacent cytoplasm (Figs 16 and 17) A basement membrane was not observed at this stage

At 8 days, the surface cells invariably formed a continuous layer with the cells in close apposition The cytoplasmic processes on the surface had the dimensions and general appearance of mesothelial microvilli (Figs 18 and 19) A basement membrane was now demonstrable beneath most cells (Fig 19) Along the borderline between adjacent cells one or more zones resembling tight junctions or desmosomes were present (Fig 20) The surface cells were frequently of a rounded shape, and they possessed a well developed endoplasmic reticulum and relatively few vesicles In other respects they exhibited the criteria of mesothelial cells characteristic microvilli tight junctions, occasionally desmosomes, and a basement membrane

In the later stages the surface cells became flatter, the microvilli longer and the endoplasmic reticulum generally less abundant (Figs 21–24) All of these changes tended to make the cells more like normal mesothelium

The presence of specialized junctional zones between adjacent cells was found to be the most reliable single criterion distinguishing mesothelial cells from fibroblasts and macrophages Special attention was therefore devoted to the cell junctions As previously mentioned, fully developed adhesion zones were not observed at 6 days At 8 and 10 days on the other hand, tight junctions were present between most neighbouring cells (Figs 25, 28, 29) In many instances several short areas of intimate contact were found along a cell junction (Fig 27) Desmosome like structures (Fig 27) or typical desmosomes (Figs 28 and 29) were less frequently observed than were the tight junctions, however, they were definitely more common during these stages of regeneration than they were in normal mesothelium In some instances, two fully developed desmosomes were found in the course of a cell contact (Fig 30)

Ocassionally, a surface cell possessed a single cilium (Fig 31) similar to the ones that were sometimes seen in normal mesothelial cells Bundles of fine cytoplasmic filaments were frequently observed in the surface cells (Fig 32) Fine filamentous structures were demonstrable within the microvilli of the new mesothelial cells, in some instances

these filaments seemed to be arranged in a circular pattern (Figs 33 and 34)

DISCUSSION

The present study has confirmed that the cells observed on the surface of peritoneal wounds in the early stages are identical in appearance with the peritoneal macrophages The observations further indicate that gradual changes take place in the morphology of these cells They soon spread out and assume a flattened shape the amount of rough surfaced endoplasmic reticulum gradually increases and from the 4th day this structure is very prominent Free ribosomes are scattered in the cytoplasm and at the same time large nucleoli are observed in most nuclei

It is interesting to note that exactly the same features have been found to distinguish growing or immature endothelial cells from those of normal endothelium (2 18 20)

At the time when these features first become evident in peritoneal wounds there are no criteria distinguishing the cells in the most superficial layer from those in the deeper parts of the wound they all resemble proliferating fibroblasts (12 17) However the surface cells gradually exhibit more numerous slender and uniform cytoplasmic processes on their free surface The spaces between these cells are reduced in size resulting in closer cell contacts 6 days after the injury small areas of cytoplasmic condensation and obliteration of the intercellular space appear along some cell junctions (Figs 16 and 17) Appearances like these may result from tangential sectioning of closely apposed cell membranes but it is equally probable that these structures represent the first stage in the formation of specialized adhesion sites (tight junctions or desmosomes)

Whether the surface cells at this stage should be classified as mesothelial cells or not may be a matter of dispute and is of minor importance In our opinion reliable criteria for this identification are not present until 8 days have passed A remarkable feature is that these changes proceed gradually and are identical in large and small wounds

Solitary cilia have previously been observed in mesothelial cells (8 11) and in differentiating fibroblasts (10 19) Fine cytoplasmic filaments similar to those depicted in Fig 32 have been described in a variety of cells including fibroblasts (12 37) and peritoneal macrophages (4) These structures are therefore not useful for the identification of the cells in question

In no instance have cells with the characteristics of mesothelial cells been observed on the surface of a wound before the 6th day The absence of mesothelial cells both among the peritoneal fluid cells and in the exudate of fresh wounds is taken as evidence against the hypothesis that mesothelial cells are detached from the peritoneum and grafted on the wound

On the other hand the observations m… are in good accordance

with the hypothesis previously presented (5) that peritoneal macrophages may differentiate to mesothelial cells

SUMMARY

Wounds of varying size were made in the parietal peritoneum of rats and studied in the electron microscope at intervals from 1 hour to 14 days The wounds were rapidly covered by peritoneal macrophages which settled in the fibrin network forming on the wound surface The observations strongly suggest that the peritoneal macrophages transformed to fibroblasts, and cells that were located in the most superficial layer gradually attained the characteristics of mesothelial cells

Even very large wounds were completely covered with new mesothelium in the course of 8 days

The observations tend to disprove the hypothesis that mesothelial cells are detached from other parts of the peritoneum and implanted in the wound as free grafts

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PENICILLIN SERUM CONCENTRATIONS IN RELATION TO EXERCISE

By

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Received 13 v 66

Previous to a mass schedule treatment of gonorrhoea in Greenland (*Tomholt & Berg* 1966) it was of importance to investigate penicillin blood levels obtained following high doses of benzylpenicillin sodium

The primary aim was to attain a sufficiently high and prolonged blood concentration which 8 hours after the injection should be at least 1 2 units pr ml This was shown to be possible by administering a single dose of 5 million units benzylpenicillin sodium supplemented with 1 G probenecid orally half an hour prior to the injection (*Schmidt & Roholt* 1965)

During these experiments a difference was noted between the penicillin serum concentrations obtained in bedridden and ambulatory patients the concentrations being lower and peak values reached later in the former group In these first experiments the difference in serum concentrations was obvious This could mean that the absorption of intramuscularly deposited solutions was dependent on the activity of the patients Later experiments in the same series indicated however that absorption of orally administered probenecid might also be a contributory factor to the difference observed It is well known that absorption of orally administered drugs varies considerably and it was therefore decided to perform an experiment without probenecid in order to decide to what extent the absorption of parenterally administered penicillin was dependent on the activity of the patient

MATERIAL AND METHODS

Six male patients in the *Department for Skin and Venereal Diseases* University Clinic of Copenhagen received on two consecutive days 5 million units benzylpenicillin sodium dissolved in 8 ml 0 5 per cent lidocaine solution (Leostesin®) by intramuscular injection All the patients had normal kidney function as shown by serum creatinine estimations

On the first experimental day penicillin was given to bedridden patients and they remained in their beds throughout the experimental period On the second day the patients were ambulatory during the experimental period Blood samples were taken ½ 1 2 4 6 and 8 hours after administration and on the second day imme

diately prior to the injection Urine was collected from the patients in the period 0–8 and 8–24 hours after injection

The concentrations of penicillin in the serum and urine samples were assayed by an agar plate method using Sarcina lutea as test organism (U.S.P. XVII)

RESULTS

Tables 1 and 2 show the serum concentrations of bedridden and ambulatory patients, respectively

It can be seen from Table 1 that in bedridden patients the average serum concentration after ½ hour is 63 units with values varying from 39 to 85 After 1 hour the average value is 69 units (52 and 108) After 2 hours the average value is 38 units (28 to 65) The values after 4, 6 and 8 hours apply to only 5 patients, patient no 3 being unable to complete the experiment on the second day Consequently, we have excluded the last three measurements of this patient from the first experimental day After 4 hours the average serum concentration is 7 9 units (4 9–13 units)

TABLE 1

Serum Penicillin Concentrations Following Intramuscular Injection of 5 Million Units Benzylpenicillin Sodium to Bedridden Patients

Subject	Serum concentrations (i u./ml) Hours after administration					
	½	1	2	4	6	8
1	39	59	31	4.9	0.77	0.12
2	85	65	31	8.9	2.4	1.0
3	64	79	65	–	–	–
4	44	52	38	13	3.2	0.92
5	69	108	37	5.5	1.5	0.40
6	76	52	28	7.2	1.7	0.42
Average	63	69	38	7.9	1.9	0.57

TABLE 2

Serum Penicillin Concentrations Following Intramuscular Injection of 5 Million Units Benzylpenicillin Sodium to Ambulatory Patients

Subject	Serum concentrations (i u./ml) Hours after administration					
	½	1	2	4	6	8
1	73	82	27	4.9	1.0	0.23
2	91	–	29	5.9	1.3	0.36
3	112	141	58			
4	87	108	36	6.9	2.0	0.67
5	130	108	31	4.3	1.0	0.36
6	113	95	42	8.9	1.8	0.41
Average	101	107	37	[illegible]	1.4	0.42

After 6 hours the figure is 1.9 (0.77–3.2) and finally the average value after 8 hours is 0.57 (0.12–1.0).

Table 2 shows the serum concentrations for the same six patients who were now ambulatory. As the experiments were carried out with 24 hours interval, blood samples were taken immediately before the second administration of penicillin in order to determine the content of penicillin, if any, due to the previous injection. However, the penicillin concentration in all sera were < 0.2 units and therefore negligible.

Table 2 shows that the average serum concentration after ½ hour is 101 units (73–130), after 1 hour the average value is 107 units (82–141 units)[1]. After 2 hours the average value is 37 units (27–58 units). The last three values are, as previously mentioned, based on only 5 patients, and are for 4 hours 6.2 units (4.3–8.9 units), for 6 hours 1.4 units (1.0–2.0 units) and for 8 hours 0.42 units (0.21–0.67 units).

TABLE 3

Urinary Penicillin Excretion Following Intramuscular Administration of 5 Million i.u. Benzylpenicillin Sodium to Bedridden and Ambulatory Patients

Subjects	Total urinary excretion in million i.u.			
	Bedridden Period after injection		Ambulatory Period after injection	
	0–8 hr	8–24 hr	0–8 hr	8–24 hr
1	3.8	0.01	2.7	0.01
2	4.4	0.05	3.7	0.02
3	3.9	0.02	2.6	< 0.01
4	4.9	0.06	3.9	0.02
5	4.2	0.12	2.9	< 0.01
6	3.2	0.01	3.5	< 0.01
Average	4.1	0.05	3.2	< 0.01

Table 3 shows the urinary excretion of penicillin in the two experimental periods of 0–8 and 8–24 hours after the injection. While bedridden, the six patients excrete an average of 4.1 million units corresponding to approximately 80 per cent (64–98 per cent) of the given dose during the first 8 hours. In the same period the ambulatory patients excrete an average of 3.2 million units corresponding to 64 per cent (52–78 per cent) of the dose given. In the following 16 hours the values are 50 000 units (1 per cent) and < 10 000 units (< 0.2 per cent), respectively.

With reference to previous investigations (*Schmidt & Roholt* 1965) where the experimental conditions for a certain group of patients were identical with those existing in the present study, it has been possible

[1] The sample from patient 2 was incorrectly assayed and has consequently been excluded.

to combine the two series so that the results cover a group of 16 ambulatory and 13 bedridden patients. The average values over a 4 hour period are shown in Table 4. The table shows for this larger group that the 2 hour values are nearly identical whereas the initial concentrations are approximately twice as high in the ambulatory group. After four hour the bedridden patients have somewhat higher serum concentrations.

TABLE 4

Average Serum Penicillin Concentrations in Man Following Intramuscular Injection of 5 Million Units Benzylpenicillin Sodium

No of subjects	Average serum concentrations i.u./ml ½ hr	1 hr	2 hr	4 hr	
16	94	108	50	10	Ambulatory
13	58	63	40	16	Bedridden

DISCUSSION

The present experiment has shown that there is a pronounced difference in penicillin serum concentrations for bedridden and ambulatory patients. Whereas the serum values at 2 hours are the same in both groups, there is a significantly higher initial concentration in the ambulatory patients. After the 2 hour values a somewhat higher concentration is observed in the bedridden group. This is probably due to differences in the rate of absorption from intramuscular administered penicillin.

In view of the increasing number of gonococcal strains with reduced sensitivity to penicillin it is of interest to increase the penicillin serum concentrations in patients. This can be achieved partly by inhibiting the renal tubular excretion by probenecid and partly by increasing the dose.

With soluble salts of penicillin the size of dose and the kidney function has till now been regarded the only important factors influencing penicillin serum concentrations, while the activity of the patient has not been given much attention.

It is surprising that it has not previously been pointed out that a relationship exists between the patient's activity and the absorption of aqueous solutions of penicillin. For streptomycin solutions, however, Riches (1954) found that side effects were more common in ambulatory than in bedridden patients and measurement of the concentrations showed that the blood streptomycin level during excercise is generally higher than that found during rest.

With regard to the urinary excretion of penicillin, the difference between our two groups is that bedridden patients excrete approximately 80 per cent of the given dose during the first 8 hours, whereas the figure for ambulatory patients is approximately 60 per cent. In the

following 16 hours, the excretion for both groups was very low It was however more than five times higher in the bedridden than in the ambulatory group

A possible explanation of the difference in total excretion of penicillin in the two groups, may be that the inactivation of penicillin and the metabolism of the organism proceed parallel and the destruction of the substance in the liver is accelerated with increased metabolism due to activity

The experiments given here should only be regarded as model experiments with an antibiotic, the excretion mechanisms of which are well known With the very large doses given here differences between the penicillin serum concentrations of bedridden and ambulatory patients is probably only of pharmacological interest With lower doses of penicillin, however, it might be of clinical therapeutic significance

During recent years, we have observed a change in sensitivity to penicillin of both staphylococci and gonococci, probably because the relatively small doses employed hitherto almost completely eradicated the most sensitive strains This would mean that in certain cases one should not only be aware of the sensitivity of the microorganism but in addition take into account the state of activity of the patients

SUMMARY

Penicillin serum concentrations following high doses of soluble benzylpenicillin have been shown to depend on the activity of the patient Ambulatory patients obtain a maximum concentration which is both higher and furthermore more rapidly obtained than that of bedridden patients The maximum serum concentrations of bedridden patients are about half that found for ambulatory patients

Two hours after the penicillin injection serum concentrations are nearly the same for both groups After two hours the bedridden patients have a somewhat higher serum concentration

The total amount of penicillin recovered in the urine is higher in bedridden patients than in ambulatory patients

This may be explained by the difference in activity leading to a higher destruction in the liver following increased metabolism

The consequences of the observations are discussed

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THE ORIGIN OF MONONUCLEAR CELLS IN HUMAN SKIN WINDOWS

By

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The acute inflammatory process still presents many controversial problems one of which is the origin of the mononuclear cells in acute inflammatory exudates. In 1955 *Rebuck & Crowley* described the skin window technique, by which a coverslip is placed on a small area of abraded skin. The exudate cells were found to adhere to the coverslip, and by replacing the coverslip at regular intervals successive populations of emigrating cells could be examined microscopically. It was claimed at the time that this method clearly established the lymphocytogenous origin of the great majority of the mononuclears since a gradual transformation of small lymphocytes into larger macrophages could be observed by examining the exudate cells on successive coverslips.

Riis (1959) using the same method also described some small mononuclears with rounded nuclei resembling lymphocytes but a regular progression of enlarging mononuclear cells was not confirmed. He suggested that the smaller cells had not adhered sufficiently well to the coverslip and therefore differed in appearance from the remaining mononuclears. *Becker & al* (1961) also studied the mononuclears in skin windows and suggested that they constituted a mixture of lymphocytogenous and histiocytogenous cells.

Recently *Volkman & Gowans* (1965a and b) in a very convincing series of experiments studied the origin of the mononuclear cells in skin windows in rats. They used a variety of techniques including labelling with tritiated thymidine, lymphocyte depletion and X-irradiation of the bone marrow, and concluded that the emigrated mononuclears arose from precursors in the bone marrow and to a lesser extent the spleen.

It is the purpose of the present experiments to re-evaluate the original hypothesis that mononuclears in human skin windows are emigrated lymphocytes. The morphology of macrophages in skin windows was re-examined but greater emphasis was placed upon their histochemical staining characteristics. In addition a simple technique was used for

obtaining mononuclears emigrating from a blood clot *in vitro*, and the morphology and histochemical properties of mononuclears emigrating in skin windows and *in vitro* were compared with those of blood lymphocytes and monocytes

MATERIAL AND METHODS

The *skin window technique* has previously been described in detail (*Rus & Wulff* 1960) The epidermis was scraped away from a small area on the forearm Minute bleeding points indicated that the papillary processes of the corium had been reached The lesion was covered by a coverslip which was protected using a cardboard square (or a square of glass) and fixed by adhesive tape The coverslips were changed hourly during the experimental period of 12 hours or longer 20 skin windows were made on 6 haematologically normal subjects 8 series were stained using the *May Grünwald Giemsa* method 6 series were stained for *a* naphthyl esterase 3 series were stained for peroxidase and 3 series were stained using Sudan black B

In vitro emigration A thick slide with a shallow central depression (22 mm in diameter) was filled with freshly drawn venous (or capillary) blood A coverslip (24 × 24 mm) was placed on top of the depression to form the roof of an incubation chamber after which the slide was left (preferably in a moist chamber) in an incubator for a period of 15 30 or 45 minutes During this time the blood clotted and cells emigrated to the coverslip In a few experiments longer incubation periods were used Afterwards the coverslip was lifted off the slide and if the clot struck to the coverslip it was carefully removed by loosening it along the edge The coverslip was finally dried in air 52 *in vitro* experiments were done using blood from 5 normal subjects The preparations were stained like the skin window preparations In addition the histochemical staining properties of lymphocytes and monocytes were examined in *blood films* and *leucocyte concentrates*

Staining reactions The peroxidase reaction was carried out according to *Sato & Sekiya* (*Darmady & Davenport* 1958) and the Sudan black B stain according to *Hayhoe* (1953) *α* naphthyl esterase was demonstrated using a coupling azo dye technique as previously reported (*Wulff* 1963) The diazonium salt used was either Fast Blue B or Fast Red TR and the preparations were fixed in 10 per cent neutral formalin saline at 4° C for 10 minutes or in formalin vapour for 4 minutes

RESULTS

Skin Window Experiments

The first cells emigrated during the second hour of the inflammatory process and the coverslips soon became paved with emigrated cells The polymorphonuclears predominated on the earliest coverslips and only few mononuclears were seen on the two and three hour preparations On the subsequent coverslips the percentage of mononuclears gradually increased and at the twelve hour stage they frequently constituted the majority of the cells (Fig 1) The morphology of the individual mononuclears varied The nuclei were kidney shaped elongated or round (Fig 2a) all transitions were seen and it was impossible to distinguish between distinct morphological types The cytoplasm was basophilic and frequently contained azurophilic granules The size of the cells also varied since both polymorphonuclears and mononuclears were smaller i e less spread out in some areas of the coverslips than in others It was however noticeable that even mononuclears with a round nucleus and a narrow cytoplasmic rim appeared as big as or bigger than the surrounding polymorphonuclears No pro-

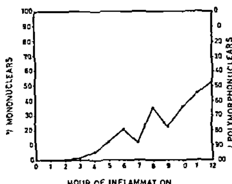


Fig 1

Differential counts of 200 cells performed on a series of skin window preparations

gressive change in the morphology of the mononuclears was observed from early to late skin window preparations

The Sudan black B stain revealed numerous distinct sudanophilic granules in all mononuclears including those on the two to four hour coverslips (Fig 4b) Only a slight increase in the sudanophilia of the cells was noticed during the twelve hour period of these experiments

The peroxidase stain revealed peroxidase activity in all mononuclears including the first to appear (Fig 3c) No increase in peroxidase activity was observed during the experimental period but the staining intensity in the individual mononuclears varied ranging from a few to numerous blue granules The degree of staining was not dependent on the nuclear morphology of the individual cells

The α naphthyl esterase reaction revealed activity in all mononuclears When Fast Blue B was used as coupling agent the cytoplasm of the mononuclears presented a diffuse black coloration whereas Fast Red TR was precipitated in numerous distinct red granules (Fig 4c) The degree of staining did not change during the experimental period

In vitro emigration During incubation leucocytes emigrated from the blood clot to the overlying coverslip After 15 minutes only a few cells were found on the coverslips but when the incubation period was extended to 30 minutes or longer, the coverslips were paved with emigrated cells comprising polymorphonuclears mononuclears and some eosinophils The mononuclears constituted about 6 per cent of the cells (range 3 12 per cent) and the percentage varied little with the length of the incubation period No mitotic figures were seen

The morphology of the individual mononuclears was variable After 15 minutes incubation the few mononuclears present were typical blood monocytes After 30 minutes incubation or longer the appearance of the emigrated mononuclears differed from that of both blood lymphocytes and monocytes Instead they closely resembled the emigrated mononuclears in skin windows (Fig 2b) Frequently the cells showed

signs of amoeboid motion which had been interrupted at the moment of air-drying

All mononuclears showed numerous sudanophilic granules in the cytoplasm (Fig 4a) and similarly all cells possessed naphthyl esterase activity using either azo dye The peroxidase reaction revealed a varying number of blue granules in the cytoplasm of the mononuclears (Fig 3b), and only a few cells were completely negative

The lymphocytes and monocytes in *blood films* and *films of leucocyte concentrates* showed the expected morphological and histochemical characteristics (Fig 2c and 3a) All lymphocytes were devoid of peroxidase activity and sudanophilia No esterase activity was seen in this cell type using Fast Blue B, but when Fast Red TR was used most lymphocytes contained a few granules The great majority (> 80 per cent) of the monocytes on the other hand possessed peroxidase activity, sudanophilic granules and strong esterase activity, when either of the two azo-dyes was used

In histochemical preparations it may prove difficult to classify a few of the mononuclear cells as either lymphocytes or monocytes It is therefore important to stress that all mononuclears possessing peroxidase activity, sudanophilia or strong esterase activity were typical large monocytes

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Fig 2

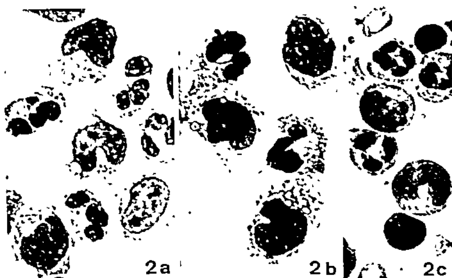
a) Mononuclear and polymorphonuclear cells emigrating in a skin window 12th hour of inflammation *b)* Mononuclear and polymorphonuclear cells emigrated from a blood clot after 45 minutes incubation *c)* Monocytes lymphocytes and polymorphonuclears in a leucocyte concentrate Stained by the May Grunwald Giemsa method

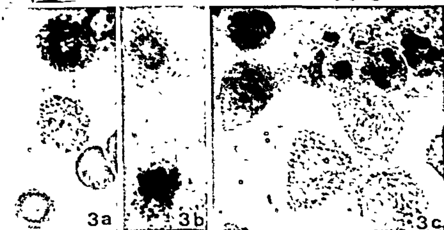
Fig 3

Peroxidase reaction according to *Sato & Sekiya* *a)* A strongly positive polymorphonuclear (above) a positive monocyte (in the middle) and two negative lymphocytes (below) from a leucocyte concentrate, *b)* A positive mononuclear (above) and a positive polymorphonuclear (below) having emigrated from a blood clot *c)* Positive mononuclear and polymorphonuclear cells in a skin window 6th hour of inflammation

Fig 4

a) Sudan black B reaction in leucocytes emigrated from a blood clot Below a mononuclear containing numerous sudanophilic granules and above a strongly positive polymorphonuclear Counterstained by *May Grünwald Giemsa* method *b)* Sudan black B reaction in leucocytes emigrating in a skin window In the centre a mononuclear cell containing numerous sudanophilic granules 3rd hour of inflammation No counterstain *c)* *a* Naphthyl esterase reaction (Fast Red TR) in cells emigrating in a skin window 12th hour of inflammation The three mononuclear cells contain numerous granules


2a
2b
2c


3a
3b
3c


4a
4b
4c

DISCUSSION

Since the pioneer works of *Metchnikoff* (1888) numerous papers have been written on the origin of the mononuclear cells in inflammatory exudates The present paper is exclusively concerned with the origin of the mononuclears in human skin windows

The similarity between the morphology of the mononuclears in the skin window experiments and the mononuclears emigrating from a blood clot clearly suggests that the former are emigrated blood cells, but does not solve the problem whether they are emigrated lymphocytes, monocytes or both The gradual transformation from lymphocyte-resembling cells to typical macrophages as described by *Rebuck & Crowley* (1955) was not confirmed A few mononuclears with round nuclei and a narrow cytoplasmic rim were seen The diameter of such cells equalled or surpassed that of the surrounding polymorphonuclears, and they therefore appeared larger than the lymphocytes in blood films However, the morphology of lymphocytes might have changed during the process of emigration Therefore it can only be concluded that the *morphology* of the mononuclears in skin windows is not more indicative of a lymphocytogenous than a monocytogenous origin It may be added that *Leder & Schomerus* (1963) observed that most emigrated mononuclears had more than one nucleolus in contrast to blood lymphocytes

It is well-known that most blood monocytes contain peroxidase and sudanophilic material, whereas all lymphocytes are devoid of these substances In the present study mononuclears emigrating in skin windows and *in vitro* possessed peroxidase activity and sudanophilia and these *histochemical* experiments therefore indicate that they are emigrated monocytes

As in previous experiments (*Wulff* 1963) the α naphthyl esterase reaction also revealed a similarity between emigrated mononuclears and monocytes, but this observation is less conclusive, as lymphocytes also possess some α-naphthyl esterase activity, when the most sensitive coupling technique is used

As mentioned in the introduction *Volkman & Gowans* (1965 a and b) convincingly showed that mononuclears emigrating in skin windows in the rat were haematogenous cells formed in the bone marrow, and consequently the blood monocytes were the obvious antecedents However, at the same time it was pointed out that the morphological distinction between lymphocytes and monocytes is unsatisfactory, and it was suggested that some of the macrophage antecedents formed in the bone marrow might not be distinguishable from blood lymphocytes That would explain *Rebuck & Crowley's* statement that some of the mononuclears emigrating in skin windows resembled lymphocytes rather than monocytes

In the present study, however, all mononuclear cells in the blood

showing the staining characteristics of emigrated mononuclears were typical monocytes and the *in vitro* experiments suggest that the morphology of the monocytes changes after emigration Otherwise the results of these experiments are in complete agreement with *Volkman & Gowans* conclusions and there is no reason for believing that mononuclears emigrating in skin windows in man and those emigrating in the rat have different origins

SUMMARY

The morphological and histochemical characteristics of mononuclears emigrating in human skin windows and in vitro were examined The morphological observations were not conclusive but the histochemical studies revealed that emigrating mononuclears with regard to sudanophilia peroxidase activity and esterase activity resembled blood monocytes Blood lymphocytes did not show these staining characteristics and the results therefore indicate a monocytogenous origin of the emigrating mononuclears

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STUDIES CONCERNING THE CYTOGENETIC RELATIONSHIP BETWEEN *IN VIVO* AND CORRESPONDING *IN VITRO* CELL POPULATIONS FROM PATIENTS WITH CHRONIC MYELOGENOUS LEUKAEMIA

By

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Received 16 vi 66

The majority of the cytogenetic investigations hitherto undertaken in patients with chronic myeloid leukaemia (CML) are based upon blood cultures The reliability of these investigations depends to a great extent upon whether the *in vitro* population gives an accurate impression of the *in vivo* conditions

Sandberg et al (1961) compared the cytogenetic patterns in short-term cultured and uncultured bone-marrow samples from nine patients suffering from leukaemia Half of the aspirated marrow from each patient was examined without preliminary culture while the other half was incubated for ten hours The cultured marrow showed a significantly higher prevalence of diploid cells than the uncultured marrow Seven out of these nine patients, however, had acute leukaemia and only two had chronic myelogenous leukaemia In one of these two patients marrow culture did not yield any cells of satisfactory quality while in the other patient the diploid and aneuploid cells were distributed in both of the marrow populations in good agreement with the expected values

In another paper, *Sandberg et al* (1962) compared the distribution of the chromosome counts in uncultured marrow and corresponding blood cultures from six patients with acute leukaemia and found by far the highest prevalence of diploid cells in the blood cultures

Fitzgerald et al (1963) found a higher prevalence of hyperdiploid and lower prevalence of hypodiploid cells in blood cultures than in uncultured marrow from patients with CML The total proportion of aneuploid cells, however, was practically uniform in the two tissues

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In order to illustrate the relationships between the *in vivo* and *in vitro* cell populations from patients with CML more exactly, the object of the present work is to compare the distribution of chromosome counts and the number and nature of the extra and absent chromosomes *in uncultured marrow specimens and corresponding blood cultures* from 15 patients with CML

MATERIAL AND METHODS

The material is composed of 17 bone marrow specimens and 17 blood cultures from 15 Ph^1 positive patients with clinically and haematologically typical CML Thirteen patients are represented by one bone marrow specimen and one blood culture each *The two sets of specimens nos 6 and 7 (Table 1) were both obtained from the same* patient with an intervening interval of seven months The interval between samples 15 and 16 which also originate from one patient, was six weeks (Table 2) The blood cultures are included in a blood culture material published previously (*Pedersen* 1966 *a b*) *The present material of bone marrow samples includes all those which* were obtained prepared according to the method described below and in which analysable cells were found The procedure of collecting the material is described in greater detail elsewhere (*Pedersen* 1966 a) Where 15 out of the 17 pairs of *samples were concerned the blood samples and bone marrow biopsy specimens* were obtained simultaneously In pairs nos 12 and 16 the blood samples were obtained five and two days respectively before the corresponding bone marrow biopsy

TABLE 1

Blood Culture (B) and Bone Marrow (M) Chromosome Counts in Ten Sample Pairs from the Untreated and Early Phase of Chronic Myelogenous Leukaemia

Phase of Disease	Sample Pair No	Tissue	Chromosome Counts					
			<45	45	46F	46L	>46	Total
Untreated	1	B	1	3	24	--		28
		M	1	2	25		--	28
	2	B	1	2	14	1	-	18
		M	--	1	17	-	-	18
	3	B	3	5	26	1	--	35
		M	--	3	32	--	--	35
	4	B		1	6	--	-	7
		M	--	--	7	--		7
	5	B		3	6		--	9
		M	1	2	6			9
	6	B	1	1	12			14
		M	2	2	9	--	1	14
Early	7	B			2		3	5
		M			3		2	5
	8	B		3	9			12
		M			12			12
	9	B	3	3	23			29
		M	1	1	26		1	29
	10	B			1		--	1
		M			1		--	1

TABLE 2

Blood Culture (B) and Bone Marrow (M) Chromosome Counts in Seven Sample Pairs from the Late Phase of Chronic Myelogenous Leukaemia

Phase of Disease	Sample Pair No	Tissue	Chromosome Counts					
			<45	45	46E	46U	>46	Total
	11	B	4	7	25	1		37
		M	2	1	34			37
	12	B		2	1	7	9	19
		M	2	3	1	8	5	19
	13	B			7	10	3	20
		M			4	5	11	20
Late	14	B			8			8
		M			8			8
	15	B	3	2	2	8	27	42
		M	2	4	5		31	42
	16	B	1	2		1	16	20
		M	2	1	1	1	15	20
	17	B					22	22
		M		1	1		20	22

The bone marrow was prepared without preliminary culture according to a modification of the method described by *Tjio & Whang* (1962) The blood was cultured 46–51 hours and the cultures prepared according to a slightly modified version of the method described by *Moorhead et al* (1960)

The procedure for selection of the mitoses for karyotype analysis the method of analysis and chromosome classification are mentioned elsewhere (*Pedersen* 1966 a) All of the mitoses in the material are Ph[1] positive

As the cytogenetic patterns in patients with CML are different at different stages of progression of the disease (*Pedersen* 1966 a) the present material was sub divided into three phase groups

1 Cultures from untreated patients all newly diagnosed (untreated phase
2 Cultures from patients who had been treated with cytostatics for less than a total of 150 days (early phase) and
3 Cultures from patients who had been treated for a total of more than 150 days (late phase)

The relationship between the cytogenetic conditions in blood cultures and corresponding bone marrow biopsy samples was investigated by comparison of

1 The distribution of the cells in the following chromosome count categories <45 45 46E 46U and >46 Cells with <45 and >46 chromosomes were each [illegible] istical [illegible] while [illegible] with [illegible]
2 Prevalence of extra chromosomes and their distribution in the various chromosome groups
3 Prevalence of absent chromosomes and their distribution in the various chromosome groups

As the frequencies of aneuploid cells and thus the extra and absent chromosomes vary greatly from one pair of specimens to another the total prevalences in the tissues compared are greatly dependent upon the number of cells analysed in the individual specimens If in a patient with a high prevalence of aneuploid cells three times as many blood culture cells as bone marrow cells are examined the

sponding samples was undertaken in the following manner From the sample with the greater number of analysed cells a number are taken equal to that contained in the corresponding smaller sample In order not to bias the selection of these cells they were included in each case in the order in which they had been analysed

RESULTS

The *distribution of the chromosome count* in the corresponding samples from each pair of samples from untreated patients and patients in the early phase of the disease appears from Table 1 while the distributions in the late phase are shown in Table 2 In Table 3 the distribution of the chromosome counts in the two tissues are compared in each phase by means of χ^2 tests None of the phases show significant differences but particularly in the samples from untreated patients and patients in the early phase the blood cultures show a tendency to higher frequency of hypodiploid cells than the bone marrow biopsy specimens and in the late phase the 46U frequency was highest in the blood cultures The total *in vitro* and *in vivo* cell populations showed definitely different distributions of the chromosome counts with significantly highest prevalence of 46U cells and a marked tendency to the highest prevalence of 45 cells *in vitro*

TABLE 3

Chromosome Count Distributions in Blood Culture (B) and Bone Marrow (M) Populations Compared by χ^2 Tests

Chromosome Count	Tissue	Phases of Disease: Untreated	Early	Late	Total
<45	B	6	3	8	17
	M	4	1	8	13
45	B	15	6	13	34
	M	10	1	10	21
46E	B	88	35	43	166
	M	96	42	54	192
46U	B	2		27	29
	M			14	14
>46	B		3	77	80
	M	1	3	82	86
Total	B	111	47	168	326
	M	111	47	168	326
P		0.40–0.[illegible]0	0.05–0.10	0.20–0.30	0.025–0.050

Nearly all of the 46U cells in the material originated from the late phase As is apparent from Table 2 the p[illegible]r of specimens no 1 in particular shows different frequency of 4[illegible]lls in the corresponding

samples Following exclusion of pair no 15 no significant differences were present between the distribution of the chromosome count in the individual phases of the disease nor after amalgamating of all the phases, where $0\,10 < P < 0\,20$

TABLE 4

Extra Chromosomes in Aneuploid Blood Culture (B) and Bone Marrow (M) Cells from the Untreated and Early Phase of Chronic Myelogenous Leukaemia

Phase of Disease	Sample Pair No	Tissue	Extra Chromosomes
Untreated	2	B	1 (21 22 Y)
	3	B	1 (19 20)
	6	M	1 (6–X 12)
Early	7	B	3 (21 22 Y)
	7	M	2 (21 22 Y)
	9	M	1 (6 X 12)

TABLE 5

Extra Chromosomes in Aneuploid Blood Culture (B) and Bone Marrow (M) Cells from the Late Phase of Chronic Myelogenous Leukaemia

Phase of Disease	Sample Pair No	Tissue	Chromosome Groups							
			21 22 Y	19 20	17 18	16	13 15	6 X 12	1 5	Total
Late	11	B	1							1
		M								–
	12	B		17					5	22
		M		18			1	1	1	21
	13	B		13				3		16
		M		16				14		30
	14	B								–
		M								
	15	B						63		63
		M	10		1		3	43	4	61
	16	B	14					18		32
		M	16				2	13		31
	17	B	35				1	97	19	152
		M	39		1		10	95	21	166

Table 4 shows the specimens in the untreated and early phases which contain *extra chromosomes* and their number and nature Table 5 gives similar information from the late phase In Table 6 the number of extra members of each chromosome group in each phase of the disease and the total number in each of the two cell populations compared are related to the other chromosomes observed in the corresponding

groups The specimens from untreated patients and patients in the early phase of the disease do not show any significant difference in frequency between the two tissues either in the individual chromosome groups or in the total frequencies In the late phase, extra (13 15) chromosomes were significantly more frequent in the bone-marrow specimens The remaining chromosome groups and similarly, the total number of extra chromosomes in the late phase do not show any significant difference in prevalence

TABLE 6

Blood Culture (B) and Bone Marrow (M) Prevalence of Extra Chromosomes Compared by χ^2 Tests Only Significant Differences Commented

		Phases of Diseases							
		Untreated		Early		Late		Total	
		extra	obs extra	extra	obs extra	extra	obs extra	extra	obs extra
21 22 Y	B	1	476	3	195	50	787	54	1458
	M		476	2	198	65	795	66	1469
19 20	B	1	440		188	30	669	31	1297
	M		444	–	188	34	666	34	1298
17 18	B		442	–	186		606		1234
	M		440		188	2	637	2	1265
16	B		219	–	93	–	332		644
	M	–	220		94		333		647
13 15	B		658	–	281	1	1001*	1	1940†
	M		664		282	16	993	16	1945
6 X 12	B		1730	–	735	181	2542	181	5007
	M	1	1734	1	736	166	2530	168	5000
1 5	B	–	1110	0	469	24	1672	24	3251
	M		1109		469	26	1673	26	3251
Total	B	2	5075	3	2147	286	7609	291	14831
	M	1	5087	3	2155	309	7633	312	14875

* † Prevalence of extra 13 15 higher in marrow than in blood culture ($0.0005 < P < 0.0010$)

The blood culture cell the ten bone marrow cells in pair no 17 and the two bone marrow cells in pair no 16 with seven (13–15) members all contain a morphologically abnormal chromosome, which has been classified in the (13–15) group The long arms of this chromosome are shorter than those of the other members of the group but considerably longer than those of the (21–22–Y) group (Fig 1) Out of the remaining four bone marrow cells with extra (13 15) chromosomes, an abnormal chromosome was found in two which was classified in the (13 15) group and the long arms of which are definitely longer than those of the other members of the group The blood cultures thus con-



Fig 1

Karyogram of bone marrow cell from specimen pair no 17 with 53 chromosomes, including extra members of chromosome pair no 1, the groups (6 X-12) and (21 22) (2 Ph¹), and lacking a (17 18) chromosome An abnormal acrocentric element is classified as a (13 15) member

tain no abnormal chromosomes and the bone-marrow specimens contain only two extra morphologically normal (13-15) members

Table 7 shows the distribution of absent chromosomes in the untreated and early phases, and Table 8 shows the distribution in the late phase In Table 9, the number of absent members in each chromosome group in each phase of the disease and the total number in each of the two cell populations compared were related to the number of chromosomes observed in the corresponding groups Specimens from untreated patients and from patients in the early phase of the disease do not show any significant difference in the individual chromosome groups nor in the total frequency of absent chromosomes in the two phase groups *Both phases, however, show a tendency to loss of more chromosomes in blood culture than in bone-marrow specimens* This tendency is apparant not only in various chromosome groups but in the total numbers for the two phases also The late phase shows a significantly higher prevalence of absent (17 18) *in vitro* than *in vivo* After amalgamating all three phases, the cultured cell population still shows a higher prevalence of absent (17 18) chromosomes and a higher total prevalence of absent chromosomes

It is apparent from Table 8 that only pair no 15 shows any difference in prevalence of absent (17 18) chromosomes of importance in the corresponding specimens If pair no 15 is excluded, neither the prevalence of absent (17-18) nor the total prevalence of absent chromo-

TABLE 7

Absent Chromosomes in Aneuploid Blood Culture (B) and Bone Marrow (M) Cells from Untreated and Early Phase of Chronic Myelogenous Leukaemia

Phase of Disease	Sample Pair No	Tissue	Chromosome Groups: 21 22 Y	19 20	17 18	16	13 15	6 X 12	1 5	Total
Untreated	1	B	2		-		1	2		5
		M	1					3		4
	2	B		1	1	1	1	1		5
		M					1			1
	3	B		-	1	1	5	7	-	14
		M	1		1	1				3
	4	B		1						1
		M								
	5	B		1		-	1	1		3
		M	1		1	1		1	1	5
	6	B	1	1		1				3
		M			2	-	1	3	-	6
Early	7	B							-	
		M								
	8	B	2					1		3
		M		-						
	9	B	3		2	1	1	4	1	12
		M	2					4	1	7
	10	B								
		M								

somes were significantly different in the two tissues ($0.60<P<0.70$ and $0.10<P<0.20$ respectively)

DISCUSSION

The cytogenetic profiles of Ph[1] positive cells vary greatly from patient to patient and in the same patient during the progress and treatment of the disease (*Ford & Clarke* 1963, *Court Brown & Tough* 1963 *Pedersen & Videbæk* 1964) In order to compare *in vivo* and *in vitro* cell populations it is essential therefore that both populations originate from the same patient and at the same time

The patient material from which the present blood culture material originates is identical with that from whom the bone marrow biopsy specimens were taken Where 15 out of the 17 pairs of specimens are concerned the blood specimens and bone marrow biopsies were obtained on the same occasion Two blood samples were withdrawn two and five days respectively prior to the corresponding bone-marrow biopsies The blood cultures were not found to differ significantly from the bone marrow specimens as regards the distribution of the chromo-

TABLE 8

Absent Chromosomes in Aneuploid Blood Culture (B) and Bone Marrow (M) Cells from the Late phase of Chronic Myelogenous Leukaemia

Phase of Disease	Sample Pair No	Tissue	Chromosome Groups 21 22 Y	19 20	17 18	16	13 15	6 X 12	1 5	Total
	11	B	2	1	1	2	1	6	1	14
		M			1		1	4		6
	12	B	1			1		8	5	15
		M	1				2	18	3	24
	13	B	10							10
		M	5		1				1	7
Late	14	B								
		M								
	15	B	2	1	39		1	1		44
		M	1	1	12	2	4	1	2	23
	16	B	1	1	4	1	2	1	1	11
		M		5	2	1	1	4		13
	17	B			22		3		1	26
		M	1		19		1		1	22

some count the prevalence of extra or absent members in the individual chromosome groups nor the total prevalence of extra or absent chromosomes in the two pairs of specimens (nos 12 and 16)

The material was subdivided into three groups Specimens from patients with high aneuploid prevalence would influence the total prevalences of these groups more than other specimens If one tissue in a group contained relatively more cells from patients with high aneuploidy frequencies that particular group would show false cytogenetic differences between the two tissues This bias was avoided by including the same number of cells in each pair of specimens From the specimen richest in cells a number of cells were taken equal to that in the corresponding specimen In order to avoid bias in the selection of these cells the cells were included in the order in which they were analysed If for example the specimen with the lesser number of cells contained 15 cells then the first 15 analysed cells were taken from the more cellular specimen

The blood cultures and the uncultured bone-marrow specimens received the same hypotonic treatment and were fixed air dried selected for analysis and analysed in the same manner

On the assumption that each pair of specimens originates from the same cell population the blood culture and bone marrow cell populations seem to be comparable to a reasonable degree This item will be further discussed later

The significantly different distribution of the chromosome count

which was found by comparison of the total cell populations of the two tissues and which is particularly due to the higher 46U prevalence in the cultured population disappears as mentioned previously when pair no 15 is excluded This pair originates from a patient rapidly deteriorating clinically and haematologically at the time of examination The cytogenetic profiles of the bone marrow and the peripheral blood may have been different at this time The marrow population may have contained cell clones not yet observable in the peripheral blood It is impossible to exclude that other patients in exacerbation of the disease may have shown corresponding differences

TABLE 9

Blood Culture (B) and Bone Marrow (M) Prevalences of Absent Chromosomes Compared by χ^2 Tests Only Significant Differences Commented

		Phases of Disease							
		Untreated		Early		Late		Total	
		abs	obs	abs	obs	abs	obs	abs	obs
21 22 Y	B	3	480	5	203	16	853	24	1536
	M	3	479	2	201	8	868	13	1548
19 20	B	4	445		188	3	702	7	1335
	M		444		188	6	706	6	1338
17 18	B	2	444	2	188	66	672	70	1304†
	M	4	444		188	35	674	39	1306
16	B	3	222	1	94	4	336	8	652
	M	2	222		94	3	336	5	652
13 15	B	8	666	1	282	7	1009	16	1957
	M	2	666		282	9	1024	11	1972
6–X 12	B	11	1741	5	740	16	2738	32	5219
	M	7	1742	4	741	27	2723	38	5206
1 5	B		1110	1	470	8	1704	9	3284
	M	1	1110	1	470	7	1706	9	3286
Total	B	31	5108	15	2165	120	8014	166	15287§
	M	19	5107	7	2164	95	8037	121	15308

† Prevalence of absent (17 18) higher in blood culture than in bone marrow (0 001<P<0 005)

§ Prevalence of absent chromosomes higher in blood culture than in bone marrow (0 010<P<0 025)

Following exclusion of pair no 15 however a tendency to higher 46U prevalence remains in the blood cultures where the prevalence of 45 cells is also slightly higher than in the bone-marrow specimens Corresponding to this the majority of blood cultures show a tendency to absence of more chromosomes than the corresponding bone marrow specimens After exclusion of pair no 15 the frequency of absent chromosomes did not prove to be significantly different in any individual chromosome group and similarly the total prevalence of absent chromosomes in the two tissues did not show any definite difference

These results may be interpreted as expressing a possible selective advantage of 45 and 46U cells over other cells *in vitro* As the tendency of blood cultures to lack relatively more chromosomes appears to affect most chromosome groups, the hypothesis presumes that absence of chromosomes is associated with selective advantages, almost regardless of which group lacks the chromosome or chromosomes This hypothesis cannot be discarded on the present basis but appears, on the other hand, to be rather improbable As cells with different genotypes are presumably phenotypically different, this explanation presumes that the *in vitro* milieu favours cells with a wide variety of different characters, to more or less the same extent

The tendency to non-specific absence of more chromosomes in the cultured than in the uncultured cell population is possibly due to artificial factors If it is presumed that the cell membranes of cultured cells are slightly more fragile than those of uncultured cells, they may be assumed to rupture more frequently during air-drying and cause non-specific chromosome loss

No chromosome group shows significantly different frequency of extra members in the tissues compared and, similarly, no definite difference between the total frequencies of extra chromosomes in the two cell populations was encountered On the other hand, the bone-marrow population contained 14 morphologically abnormal acrocentric chromosomes which were larger or smaller than the members of the (13–15) group as compared with the one acrocentric chromosome of abnormal morphology in the cultured population This difference is significant ($P<0\ 0005$) and may indicate a negative *in vitro* selection of cells with such abnormal elements in the karyotype

The results of the present investigation thus appear to indicate that Ph[1]-positive cells from patients with CML and with exclusively normal chromosomes in the karyotype are not exposed to *in vitro* selection on a cytogenetic basis

These results are apparently not in agreement with those obtained by *Sandberg et al* (1961, 1962) who demonstrated *in vitro* selection in favour of diploid and at the expense of aneuploid cells This selection, however was demonstrated exclusively in materials from patients with acute leukaemia The fact that leukocytes from patients with acute leukaemia grow considerably worse *in vitro* than blood cultures from patients with CML (*Sandberg et al* 1962, *Fitzgerald et al* 1964) shows, however, that the two leukaemic cell populations follow entirely different rules *in vitro*

Fitzgerald et al (1963), in contrast to the present results, found a lower frequency of hypodiploid cells and a higher frequency of hyperdiploid cells in blood cultures than in uncultured bone-marrow They found a prevalence of hypodiploid cells of 14 3 per cent which is in good agreement with the figure of 15 6 per cent in the present work On the other hand, the frequency of hypodiploid bone-marrow cells in the

material of *Fitzgerald et al* was considerably higher (21 8 per cent) than in the present material (10 4 per cent) This difference may be due to an artificial greater chromosome loss in the bone marrow material of *Fitzgerald et al* Such a chromosome loss may possibly explain the observation that the bone-marrow population showed a lower prevalence of hyperdiploid cells than the blood cultures

SUMMARY

In 15 patients with clinically and haematologically typical chronic myelogenous leukaemia cytogenetic investigation of blood cultures and uncultured bone marrow biopsy specimens was undertaken with the object of comparing the cytogenetic patterns A total of 17 blood cultures and 17 bone marrow specimens were examined In 15 pairs of specimens the corresponding specimens were obtained simultaneously while in two pairs the blood for culture was withdrawn two and five days respectively before the bone-marrow biopsy was undertaken The material comprised a total of 376 Ph[1] positive blood culture cells and 326 Ph[1] positive bone-marrow cells

The cultured cell population contained more unbalanced diploid cells than the uncultured population A considerable fraction of the difference, however was due to one particular pair of specimens After exclusion of this pair no significant difference was observed but a tendency to higher frequency of hypodiploid and unbalanced diploid cells remained however in the *in vitro* population

The blood culture cells showed the greatest absence of (17–18) members Almost the entire difference in the tissues compared however was due to the pair of specimens mentioned above Following exclusion of this pair a non specific tendency to absence of more chromosomes in the cultured cell population remained although no significant difference was shown in any chromosome group

The bone marrow population contained significantly more cells with morphologically abnormal acrocentric chromosomes all of which were larger than the members of the (21 22 Y) group and larger or smaller than chromosomes of the (13 15) group The number of morphologically normal extra members of the individual chromosome groups and also the total number of extra chromosomes did not show any *significant difference in the two tissues*

These results yield no evidence to presume that any significant *in vitro* selection occurs on a cytogenetic basis among cells without morphologically abnormal chromosomes in the karyotype The tendency to absence of a greater number of chromosomes in the blood culture population is possibly due to artificial chromosome loss

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AUTOLOGOUS *VERSUS* HOMOLOGOUS CULTURE MEDIA IN BLOOD CULTURES FROM PATIENTS WITH CHRONIC MYELOGENOUS LEUKAEMIA COMPARISON OF THE CYTOGENETIC PICTURES

By

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During the hectic cytogenetic research activity in recent years *in vitro* culture of leukocytes has become the diagnostic aid of choice The great majority of cytogenetic investigations in patients with chronic myelogenous leukaemia have been carried out by the method described by *Moorhead et al* (1960) frequently with one or other modification An important ingredient of the culture medium which this method requires is human plasma or serum The literature available suggests that certain authors elect to use the patient s own plasma (autologous medium) while others employ serum from healthy donors (homologous medium) most frequently of blood type AB and still others employ both media in the same culture There are however no investigations to illustrate whether the two media result in cytogenetically different conditions in the corresponding blood cultures

The object of the present work is to compare the cytogenetic conditions in leukocytes from patients with chronic myelogenous leukaemia grown in the two media The comparison will include not only the prevalences of Ph[1] positive cells in cultures grown with phytohaemagglutinin but also the chromosome count distribution in the Ph positive cells and the distribution of extra and absent chromosomes in the aneuploid cells of this population in the chromosome groups laid down by the Denver Convention In addition the chromosome counts and the distribution of the extra and absent chromosomes were investigated in Ph[1] negative cells from the cultures of the material for comparison with the conditions in the Ph[1] positive population

MATERIAL AND METHODS

The present material is included in a previously published blood culture material from patients with clinically and haematological all p cal chron c m elogenous

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leukaemia (*Pedersen* 1966) and comprises all cultures in which the leukocytes were incubated both with autologous and homologous media

Culture was undertaken according to a modification of the method described by *Moorhead et al* (1960) 20 ml heparinized venous blood is allowed to sediment spontaneously at room temperature for ½ to 1 hour The leukocyte count in the supernatant fluid is determined TC 199 (Glaxo) is added and autologous or homologous AB serum until the culture medium consists of 70 per cent TC 199 and 30 per cent plasma or serum and contains approximately 1 5 million leukocytes per ml Immediately prior to incubation 0 1 ml phytohaemagglutinin per 5 ml suspension is added In some cultures some of the leukocytes were grown without phytohaemagglutinin The period of culture is 46–51 hours Four hours prior to harvesting the cultures 0 1 ml of 0 04 per cent Colcémid (Ciba) per 5 ml blood culture is added After the conclusion of incubation the cells are centrifuged out by 800 rotations per minute for ten minutes and are treated for 15 minutes with 0 95 per cent sodium citrate in redistilled water After centrifuging out of the cells and removal of the supernatant fluid by suction the cells are fixed in a mixture of methyl alcohol and glacial acetic acid in the proportion 1 3

Spreading of the mitoses is obtained by careful evaporation of the fixative over a spirit flame after which the preparations are stained by Giemsa s method

The homologous serum employed as the culture medium originated from healthy donors of blood type AB Immediately after withdrawal from the donors the blood was set aside to coagulate at room temperature for approximately 24 hours after which the serum was sterilized by filtering and frozen

As mentioned previously, the object of the investigation is to compare the Ph¹ prevalence distribution of the chromosome count and distribution of the extra and

numbers of analysed cells a number of cells equal to that in the poorly represented medium was included from the better represented medium To avoid bias on selection the cells from the better represented medium were included in the order in which they were analysed The Ph¹ prevalence was compared in 47 cultures all of which were grown with phytohaemagglutinin, from 26 patients

2) The distribution of chromosome count was investigated in the Ph¹ positive and Ph¹ negative cells Concerning both categories a number of cells was taken from the better represented medium equal to that in the poorly represented medium Here also the cells from the better represented medium are included in the order in which the cytogenetic analysis had been performed The distribution of the chromosome count was compared in all pairs of cultures which contained Ph¹ positive cells and all pairs of cultures which contained Ph¹ negative cells 41 Ph¹ positive pairs of cultures from 26 patients and 38 Ph¹ negative pairs of cultures from 24 patients respectively grown both with and without phytohaemagglutinin

3) *The distribution of the extra and absent chromosomes in the aneuploid cells* in the Denver Convention chromosome groups was investigated and compared in the cell population included in (2)

The material includes only cells which could definitely be classified as Ph¹ positive or Ph¹ negative Ph¹ negative cells lacking one or more small acrocentric chromosomes may have lost Ph¹ during culture or preparation of the slides Such cells are not interpreted as definitely Ph¹ negative and thus are not included in the present material

RESULTS

In each of 47 pairs of cultures the frequencies of Ph¹-positive and Ph¹ negative cells from homologous and autologous culture media were compared by means of χ^2 tests In 42 pairs P was found to be > 0.05 The results in the remaining five pairs are shown in Table 1 where the autologous medium in pair no 1 contained the highest prevalence of Ph¹-positive cells while the homologous medium in the remaining four

pairs showed the relatively greatest frequency of Ph^1-positive cells The patient from whom the cultures in pair no 1 originated was at the time of investigation, a newly diagnosed case and treatment with cytostatics had not been instituted Pair no 3 originated from a patient who had been treated with a total dose of 925 r applied to the spleen three months previously and in whom cytostatic treatment had been commenced on the day before the cytogenetic investigation The remaining three pairs of cultures originated from patients in the last phase of the disease, i e at the time of investigation they had been subjected to cytostatic treatment for more than a total of 150 days (*Pedersen* 1966) Pairs of cultures nos 4 and 5 originated from the same patient with an intervening interval of 25 days

TABLE 1

Significantly Different Distributions of Ph^1-Positive and Ph^1 Negative Cells in Autologous and Homologous Culture Medium in Five Blood Cultures from Four Patients with Chronic Myelogenous Leukaemia

Cultures	Culture Medium	Ph^1-Pos Cells	Ph^1-Neg Cells	Total	P
1	Homologous	9	6	15	0 008
	Autologous	15		15	
2	Homologous	16		16	0 022
	Autologous	11	5	16	
3	Homologous	18	5	23	0 033
	Autologous	11	12	23	
4	Homologous	13	10	23	0 007
	Autologous	4	19	23	
5	Homologous	13	8	21	0 002
	Aut logous	3	18	21	

Table 2 shows the number of Ph^1 positive and Ph^1 negative cells from the two methods of culture in the 42 pairs of cultures none of which show any significantly different distribution The homologous medium contains a relatively greater proportion of Ph^1 positive cells than the autologous medium but the difference is not significant

The cultures in Table 2 are subdivided into two groups according to the treatment situation at the time of investigation One group comprises 24 cultures from 16 patients who were receiving cytostatic therapy at the time of investigation or had concluded the final series of treatment less than ten days before the cytogenetic investigation The second group comprises 18 cultures from 15 patients in whom such therapy had not yet been commenced or in whom the last period of therapy was concluded more than ten days before the time of investigation In both groups the cell population from the homologous medium shows a slightly higher prevalence of Ph^1 positive cells than the popula

tion which was cultured in autologous medium but the difference is not significant ($0.20<P<0.30$ and $0.30<P<0.40$ respectively)

TABLE 2

Forty Two Blood Cultures Each with Randomly Distributed Prevalences of Ph[1] Positive and Ph[1] Negative Cells Comparison of Total Prevalences in Autologous and Homologous Culture Medium

Culture Medium	No of Cultures	Ph[1] Pos Cells	Ph[1] Neg Cells	Total	P
Homologous	42	355	378	733	0.10–0.20
Autologous	42	326	407	733	
Total		681	785	1466	

The cultures in Table 2 were in addition subdivided according to the phase of disease in which the patients were at the time of investigation In six cultures from six untreated patients $0.30<P<0.40$ was found In 20 cultures from 15 treated patients in the early phase of the disease i e from patients who had been subjected to treatment for *less* than 150 days at the time of investigation (*Pedersen* 1966) $0.10<P<0.20$ was found In both groups the cell population which was cultured in the homologous medium contained most Ph[1] positive cells In 13 cultures from nine patients in the late phase of the disease i e patients in whom cytostatic therapy had continued for more than a total of 150 days $0.20<P<0.30$ was found However in contrast to the other two phase groups the proportion of Ph[1] positive cells was found to be highest in the population cultured in autologous medium One culture from a patient who had been treated practically exclusively with radiation of the spleen and two cultures from a patient in whom the data regarding treatment were not fully elucidated could not be classified as regards the phase of the disease and these patients are therefore not included in the abovementioned three phase groups

The distribution of the chromosome counts in the cell populations compared are shown in Table 3 The composition of the culture media does not appear to result in any difference in the distribution of the chromosome count in the Ph[1] positive or Ph[1] negative cells

The distribution of extra and absent chromosomes in aneuploid cells in the chromosome groups is shown in Table 4 The Ph[1]-positive cell populations resulting from the two methods of culture show practically uniform distribution and more or less identical prevalences of extra and absent members in each individual chromosome group In the Ph[1] negative population too few extra chromosomes were found for the distribution in the chromosome groups to be calculated statistically The culture media do not however appear to result in different distributions and similarly the total number of extra chromosomes was

TABLE 3

Chromosome Count Distributions of Ph¹ Positive and Ph¹-Negative Cells
Comparison of Cell Populations from Autologous and Homologous Culture Medium

Cells	Culture Medium	No. of Cultures	Chromosome Counts < 45	45	46F	46U	> 46	Total	P
Ph¹ Pos	Homologous	41	21	39	214	29	124	427	0.60–0.70
	Autologous	41	21	53	202	29	122	427	
Ph¹ Neg	Homologous	38	8	30	316	11	3	368	0.30–0.40
	Autologous	38	13	32	303	11	9	368	

TABLE 4

Distribution of Extra and Absent Chromosomes in Aneuploid Ph¹ Positive and Ph¹-Negative Cells
Comparison of Cell Populations from Autologous and Homologous Culture Medium

Cells	Chromosomes	Culture Medium	Chromosome Groups 21–22, Y	19–20	17–18	16	13–15	6–X–12	1–5	Total	P
Ph¹ Positive	Extra	Homologous	78	17		7	10	279	17	408	0.90–0.95
		Autologous	90	16	1	8	10	284	22	431	
	Absent	Homologous	36	19	91	12	19	26	6	209	0.70–0.80
		Autologous	29	22	96	7	18	23	10	205	
Ph¹ Negative	Extra	Homologous	4	2	4	1		7		18	*
		Autologous	5	1	3	1		11		21	
	Absent	Homologous	*	5	8	2	17	29	3	64	0.20–0.25
		Autologous	*	8	8	6	8	39	11	80	

practically identical The Ph^1-negative cell population which was cultured in autologous plasma shows more absent chromosomes than the corresponding homologous population The difference, however, is not significant ($0.20<P<0.30$) Similarly, the distribution of the absent chromosomes was not significantly different in the populations compared

DISCUSSION

The great majority of the cytogenetic investigations hitherto carried out on patients with chronic myelogenous leukaemia are based on analyses of blood cultures With a few exceptions, the blood cultures were performed by the method described by *Moorhead et al* (1960) or modified versions of this method Only a few authors have published details of their methods of culture As far as can be judged from the basis of this information, autologous culture media (*Bowen & Lee* 1963, *Luers et al* 1963, *Jung et al* 1963, *Goh & Swisher* 1964) and homologous culture media (*Sandberg et al* 1962, *Levan et al* 1963, *Engel et al* 1964, *Makino & Awa* 1964, *de Grouchy et al* 1965) appear to have been employed to a more or less uniform extent Certain authors state that they have employed both media (*Nowell & Hungerford* 1961, *Court Brown & Tough* 1963)

Nowell & Hungerford (1961) noticed that the Ph^1-positive cells appear to grow better in a homologous medium than in an autologous medium As far as the present author is aware, no systematic comparison between the cell populations cultured in the two media has been performed

Each of the blood cultures in the present material was grown both in autologous and in homologous medium Thus, each pair of cultures originates from the same patient, at the same time and, apart from the difference in the culture medium, has received the same *in vitro* treatment The mitoses in the cell populations compared were selected for analysis according to the same principle (*Pedersen* 1966) and were analysed in the same manner If the samples in the individual pairs of cultures had included different numbers of cells analysed, the results of the investigation might have shown false differences between the populations compared If, for example, in blood cultures with high prevalence of aneuploid cells, twice as many mitoses were analysed from the homologous culture medium as from the autologous medium and had the relationship between the numbers of analysed cells in other cultures with low aneuploid prevalences been reversed, the cells from the homologous medium would have shown higher average prevalence of aneuploid cells than the cell population cultured in the autologous medium other factors being equal In order to avoid bias, the two culture media in each culture were represented by equally many cells because as many cells were included from the better represented

medium as were comprised in the poorly represented medium These cells were selected in the order in which they had been analysed The cell populations compared may thus on this basis be reasonably considered to be comparable

Some of the blood cultures compared are grown with and others without phytohaemagglutinin There is however, no evidence suggesting that phytohaemagglutinin has any direct or indirect influence on the karyotype profiles of Ph^1 positive of Ph^1 negative cells from patients with chronic myelogenous leukaemia

In evaluation of the present results it must however, be noted that the leukocytes were not washed prior to placing in the culture media This implies that in cultures with homologous medium there has been a slight admixture of the patient s own plasma

The present results suggest that neither in the Ph^1 positive nor the Ph^1 negative population does the culture medium result in selection of cells with definite karyotypes as the distribution of the chromosome counts in the cell populations compared does not show any definite difference and similary, the distribution of the extra and absent chromosomes in the aneuploid cells of the population is not definitely different In the homologous culture medium the prevalences of Ph^1 positive cells appear to be slightly higher than in the autologous medium The difference is independent of the situation as regards treatment at the time of investigation On the other hand however, the autologous medium at the late phase appears to contain relatively more Ph^1 positive cells which is the opposite state of affairs to that in the early phase of the disease As the P value in all of the groups is quite high no definite significance can be attributed to these observations The results presented suggest therefore that the autologous or homologous character of the human culture medium has no decisive influence upon the cytogenetic conditions in blood cultures from patients with chronic myelogenous leukaemia

SUMMARY

In a blood culture material from patients with clinically and haematologically typical *chronic myelogenous leukaemia* the cytogenetic relationships were compared in cultures the human culture media of which were the patient s own plasma (autologous medium) and AB serum from healthy donors (homologous medium) For each culture grown in autologous medium there was a corresponding culture grown in homologous medium and originating from the same patient obtained at the same time and apart from the different human media grown under the same conditions *in vitro* Further the mitoses in each pair of cultures were selected for analysis according to identical principles and analysed in the same manner The corresponding cultures comprise equal numbers of analysed cells

The prevalences of Ph^1 positive cells w [illegible] in the two media

in 47 pairs of cultures grown with phytohaemagglutinin The Ph[1] prevalences in the two media varied greatly from culture to culture and were significantly different in five pairs of cultures In the remaining 42 pairs of cultures, the average prevalence of Ph[1] positive cells was slightly higher in the homologous than in the autologous cell population The difference was not significant either in the total material nor in the individual phase or treatment groups The distributions of the chromosome numbers in the Ph[1] positive and Ph[1] negative cells which were investigated in 41 and 38 pairs of cultures, respectively did not show any definite differences between the populations compared Similarly, the distribution of extra and absent chromosomes in the aneuploid cells of these populations was not found to be significantly different The present investigation yields no evidence to presume that autologous or homologous culture media result in cytogenetic differences in the cell populations in the corresponding blood cultures

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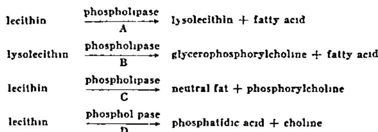
PHOSPHOLIPASE A PRODUCTION IN *STAPHYLOCOCCUS AUREUS*

By

BORJE NYGREN JAN HOBORN and PER WÅHLEN

Received 3 iii 66

Phospholipases are enzymes which hydrolyse lecithin and other phospholipides The four defined reaction types provide the foundation for the designation of these enzymes as phospholipase A B, C and D In relation to lecithin the hydrolysis scheme is realized as follows



Some information is available on the capacity of bacteria to form each of these enzymes but the major portion is in reference to phospholipase C For example this enzyme is produced by certain clostridia and aerobic spore formers (*Macfarlane & Knight* 1941)

A characteristic of phospholipase C forming bacteria is their capacity to cause opalescence when growing on egg yolk medium Even certain staphylococcal strains give rise to such an opalescence (*Gillespie & Alder* 1952) This phenomenon has been correlated to certain characteristics of the strains *i e* virulence (*Alder et al* 1953 *Reid & Wilson* 1959) or phage type and sensitivity for antibiotics (*Jessen et al* 1959) The factor which induces the type of egg yolk opacity reaction given by certain staphylococci has been shown by *Shah & Wilson* (1963 1965) to be a lipase That the formation of phospholipase D is not necessarily related to haemolytic activity of a bacterial strain was demonstrated by *Fossum & Hoyem* in 1963

The intent of the present investigation was to try to establish whether or not *Staphylococcus aureus* has the capacity to form phospholipase A

MATERIAL

For the experiments 97 strains of coagulase positive staphylococci with various phage patterns were selected. Of these 95 were isolates from clinical samples sent to the Department of Clinical Bacteriology, Gothenburg. The other two strains were the internationally employed enterotoxin forming reference strains designated No 196 and No S 6.*

as the *fluid base medium*. To this base medium lecithin was added at 0.4 per cent w/v concentration to constitute the *fluid lecithin medium*.

Lecithin was extracted from the yolks of hen's eggs with *Pangborn's* (1951) method. The product thus obtained is chromatographically free of lysolecithin. As a source of *phospholipase A* was employed rattlesnake (Crotalus adamanteus) venom distributed by Sigma Chemical Co., St. Louis, Mo. The *lysolecithin* used was also provided by Sigma.

METHODS

Cultivation. The 97 strains were pre-cultivated in meat extract broth and then grown on egg yolk broth in testtubes. These cultures were incubated for 2 days at 37° C and platinum loop transfer made to egg yolk plates (*Nygren* 1962), which were incubated for 24 hours at 37° C. Thereafter transfer to another egg yolk plate was made from a typical colony of each strain and the inoculum spread evenly over the plate. After 24 hour's incubation the bacterial mass was suspended in 2 ml of the described fluid base medium, the suspension removed and centrifuged as before. Again the supernate was removed and the sediment mixed with 2 ml of fluid base medium. These suspensions were further diluted with the medium until an extinction of 0.1 at 576 mμ was obtained with a Beckman DB spectrophotometer with the medium used as a blank. An inoculum of 0.25 ml of newly prepared suspensions was employed in 5 ml of the fluid lecithin medium. The cultures were incubated for 2 days at 37° C.

Extraction. Five ml of ether was added per fluid lecithin medium culture. After manual shaking the suspensions were centrifuged at 3000 rpm for 10 min with the formation of 3 layers—an upper ether phase, a water phase and a sediment of bacteria. The ether phase was pipetted off and saved and the remainder shaken again with a new aliquot of ether. Then the mixture was recentrifuged and the ether phase again removed. This procedure was repeated until 8 ether phases had been obtained. Those for each strain were pooled and evaporated first with a stream of cotton filtered air passing through the flask and then by lyophilization. Finally the water phase was also pipetted off, lyophilized and stored in a dessicator under vacuum.

Thin layer chromatography. Portions of the lyophilized phases were dissolved in their original solvents (ether or water) and applied on a silica gel G sheet with lecithin (in ethanol) and lysolecithin (in water) as controls. The eluant employed was a mixture of chloroform, methanol and water (65 : 35 : 4 parts). The elution time was 60 min and the temperature 24.0° C. For purposes of comparison similar chromatographic separations were run of the ether and water phases obtained from an uninoculated tube of medium with lecithin and from a similar tube to which 1 mg of phospholipase A had been added and which had thereafter been incubated for 2 days at 37° C. The preparations of the uninoculated media were worked up exactly as those of the cultures.

demonstrated to contain lysolecithin was chosen for infra red spectrophotometric

* Strains No 196 and No S 6 were kindly furnished by Dr *H. Hallander*, The Institution for Bacteriology and Hygiene, Uppsala, Sweden.

x	x	x	x	x	x	x	x	x	x
E	W	F	W	F	W	F	W	F	W
92 strains		5 strains		Medium		Medium + phospholipase A		Lecithin	Lysolecithin
Staphylococcus aureus								Control	

Fig 1

Schematic representation of thin layer chromatograms of the ether (E) and water (W) phases of cultures of 97 strains of *Staphylococcus aureus* x = start

analysis The red ssolved water phase res due f th s culture was chromatographi

spectrophotometer

RESULTS

The resuls of the chromatographic analyses are exemplified in Fig 1 Of the 97 tested strains 92 (included no 196 and no S6) did not form phospholipase A—that is to say no influence which could be related to this enzyme was demonstrated with the employed methods Chromatograms of the ether and water phases of these 92 strains showed patterns indistinguishable from those of the uninoculated medium without phospholipase A No spot occurred at the site comparable to the level of the control lysolecithin Contrarily five strains demonstrated the presence of a substance in the water phase at this site Furthermore the over all patterns for these five strains closely resembled the patterns for the ether and water phases of the uninoculated medium treated with phospholipase A

That the spot in the chromatograms of the water phases from these 5 staphylococcal cultures represented lysolecithin was tested by IR spectro-photometry Fig 2 consists of the IR spectrogram for the eluted material after chromatographic separation of the water phase of one of these five strains and the IR spectrogram obtained with the Sigma preparation of lysolecithin There good concordance of these

MATERIAL

For the experiments 97 strains of coagulase positive staphylococci with various phage patterns were selected Of these 95 were isolates from clinical samples sent to the Department of Clinical Bacteriology, Goteborg The other two strains were the internationally employed enterotoxin forming reference strains designated No 196 and No S 6 *

The bacterial cultivations were carried out using a fluid medium prepared principally according to *Halbert* (1958) with modifications suggested by *Holm & Falsen* (1966) and composed of dialysed yeast extract and trypsin digested proteose-peptone aspargine cystein hydrochloride sodium citrate sodium chloride and sodium bicarbonate glucose and monopotassium phosphate This mixture will be referred to as the *fluid base medium* To this base medium lecithin was added at 0 4 per cent w/v concentration to constitute the *fluid lecithin medium*

Lecithin was extracted from the yolks of hen s eggs with *Pangborn*'s (1951) method The product thus obtained is chromatographically free of lysolecithin As a source of *phospholipase* A was employed rattlesnake (Crotalus adamanteus) venom distributed by Sigma Chemical Co St Louis Mo The *lysolecithin* used was also provided by Sigma

METHODS

Cultivation The 97 *strains were pre-cultivated in meat extract broth and then* grown on egg yolk broth in testtubes These cultures were incubated for 2 days at 37° C and platinum loop transfer made to egg yolk plates (*Nygren* 1962) which were incubated for 24 hours at 37° C Thereafter transfer to another egg yolk plate was made from a typical colony of each strain and the inoculum spread evenly over the plate After 24 hour s incubation the bacterial mass was suspended in 2 ml of the described fluid base medium the suspension removed and centrifuged as before Again the supernate was removed and the sediment mixed with 2 ml of fluid base medium These suspensions were further diluted with the medium until an extinction of 0 1 at 576 mμ was obtained with a Beckman DB spectrophotometer with the medium used as a blank An inoculum of 0 25 ml of newly prepared suspensions was employed in 5 ml of the fluid lecithin medium The cultures were incubated for 2 days at 37° C

Extraction Five ml of ether was added per fluid lecithin medium culture After manual shaking the suspensions were centrifuged at 3000 rpm for 10 min with the formation of 3 layers—an upper ether phase a water phase and a sediment of bacteria The ether phase was pipetted off and saved and the remainder shaken again with a new aliquot of ether Then the mixture was recentrifuged and the ether phase again removed This procedure was repeated until 8 ether phases had been obtained Those for each strain were pooled and evaporated first with a stream of cotton filtered air passing through the flask and then by lyophilization Finally the water phase was also pipetted off lyophilized and stored in a dessicator under vacuum

Thin layer chromatography Portions of the lyophilized phases were dissolved in their original solvents ether or water and applied on a silica gel G sheet with lecithin (in ethanol) and lysolecithin (in water) as controls The eluant employed was a mixture of chloroform methanol and water (65 35 4 parts) The elution time was 60 min and the temperature 24 0° C For purposes of comparison similar chromatographic separations were run of the ether and water phases obtained from an uninoculated tube of medium with lecithin and from a similar tube to which 1 mg of phospholipase A had been added and which had thereafter been incubated for 2 days at 37° C The preparations of the uninoculated media were worked up exactly as those of the cultures

The chromatograms were developed for phosphorus with a liquid spray consisting of 70 per cent perchloric acid 1 M hydrochlorid acid and 4 per cent ammonium molybdate in 5 10 25 parts (*Hanes & Isherwood* 1949) After the spray application the plates were exposed to UV light

Infra red spectrophotometry One of the cultures which was chromatographically demonstrated to contain lysolecithin was chosen for infra red spectrophotometric

* Strains No 196 and No S 6 were kindly furnished by Dr *H Hallander* The Institution for Bacteriology and Hygiene Uppsala Sweden

phospholipases is spread throughout organic tissue it is not surprising that numerous attempts have been made to clarify the circumstances related to the capacity of bacteria to form these enzymes For surveys the reader is directed to *Hayaishi & Kornberg* (1955) *Ammon & Dirscherl* (1959) and *Esselmann & Liu* (1961)

SUMMARY

Ninetyseven strains of *Staphylococcus aureus* were investigated with respect to their capacity to produce phospholipase A The method em ployed in this investigation a combination of thin layer chromatography and infra red spectroscopy, permitted a direct identification of the products formed by the hydrolytic action of this enzyme Five of the tested strains were shown to produce phospholipase A

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CLASSIFICATION OF HUMAN SERUM PREALBUMINS AFTER STARCH GEL ELECTROPHORESIS

By

MAGNE K FAGERHOL and MIKAEL BRAEND

Received 9 iii 66

In a recent report (3) we described genetic variation in a system of serum proteins (Pi) migrating faster than albumin in acidic starch gels The present report deals with further characteristics of the Pi proteins primarily their relation to other proteins in the prealbumin region[1]

MATERIAL AND METHODS

Normal human sera Sera from healthy blood donors were used soon after the bleeding or after storage at —22° C

Anti human sera These were obtained from rabbits a goat and a horse after immunization with pooled normal human serum

Starch gel electrophoresis A modification of *Poulik* s (8) horizontal discontinuous system was used

Pi protein This was prepared by elution from starch gels using the method described by *Hoerman et al* (5)

Agar electrophoresis and specific staining methods This was performed as described by *Grabar & Burtin* (4)

Perchloric acid extract of human serum The extraction was performed as described by *de Vaux St Cyr et al* (12)

RESULTS

1 *Electrophoretic Studies*

Starch gel electrophoresis at alkaline or neutral pH usually reveals two protein zones in front of the albumin These zones together with a third one are shown in Fig 1 The third zone consists of the Pi proteins which at pH 7 7 or lower migrate in front of the albumin When the pH of the gel is decreased further towards 5, however more protein zones emerge from the albumin front At the same time the appearance of the Pi proteins changes resulting in a minimum of three bands (3)

The optimal electrophoretic conditions for the various proteins in the prealbumin region are different Consequently it has been impos-

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[1] In the report (ref no 3) the term Pr proteins was used It has now been shown (*Fagerhol & Laurell* in preparation) that this protein is identical with α_1 antitrypsin and the term Pi (protease inhibitor) has been chosen for the system

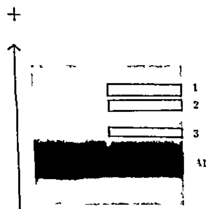


Fig 1

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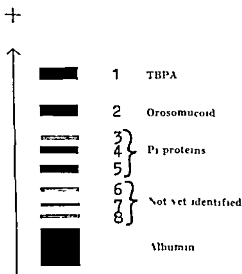


Fig 2

Schematic drawing of protein zones in the prealbumin region in starch gels

sible to obtain distinct zones for all of them in the same gel A schematic drawing of the prealbumin region at various pH is therefore presented in Fig 2

The protein band marked No 1 is one of the three thyroxine binding proteins of human serum and is called thyroxine binding prealbumin or TBPA (7) Band No 2 is orosomucoid or α_1 acid glycoprotein (10) which has been shown to be polymorphic for reference see *Schmid et*

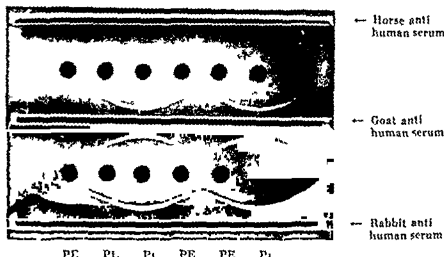


Fig 3

Photograph of an agar gel showing precipitation lines obtained by testing perchloric acid extract of human serum (PE) and Pi proteins (Pi) against horse goat and rabbit anti human sera

al 1965) Between the Pi bands and the albumin we regularly find three protein zones but these are considerably weaker than the Pi zones Still more bands but even fainter have been observed between band No 8 and albumin but these are not included in the drawing The Pi proteins and the three zones behind may correspond to the α_1 globulin B and α_1 globulin C of *Poulik & Smithies* (9) The α_1 globulin B being the quantitatively major component of α_1 globulin was unresolved from albumin in their one dimensional starch gel electrophoresis The principal electrophoretic α_1 globulin of human serum is α_1 antitrypsin which has been found to be polymorphic (for reference see *Axelsson & Laurell* 1965)

2 *Immunological Studies*

Fig 3 is a photograph of an agar gel where Pi proteins eluted from starch gels and perchloric acid extract of human serum (PE) were tested against anti human sera from horse goat and rabbit Serum from the horse gave a precipitate with PE of which orosomucoid is the main component (12) Serum from the goat precipitated the Pi proteins while serum from the rabbit precipitated both the Pi proteins and at least two proteins in PE

There was a clear reaction of non identity between the proteins of PE and the Pi proteins It is therefore assumed that the Pi proteins are not soluble in perchloric acid at least not in immunologically active form

3 *Staining Reactions*

The Pi proteins stain like glycoproteins as the reactions were positive with the periodic acid Schiff reaction, the periodic acid formazan reaction and the periodic acid NADI reaction after electrophoresis in agar gels The Pi proteins gave negative reactions with the mercury Schiff method and with Sudan black B and oil red O stains

DISCUSSION

Classification of serum proteins implies the use of methods capable of sorting out molecules differing in one or more respects The term prealbumins has been used for proteins migrating in front of the albumin in electrophoresis at alkaline pH Since the Pi proteins migrate slightly faster than albumin at pH 7 7 (Fig 1) we found it appropriate to classify them as prealbumins The symbols Pi for the system and F, M and S for the band patterns were chosen in accordance with suggestions for nomenclature of polymorphic protein systems (2) We prefer, however, to use the term prealbumins as a general term for proteins migrating faster than albumin in gel electrophoresis even when techniques with very high resolving power are used Some of the prealbumins have been given more characteristic names or symbols such as orosomucoid (13) and TBPA (6) When the other prealbumins have been studied in greater detail more descriptive names may eventually be taken into use for these also

SUMMARY

Starch gel electrophoresis of human serum at pH 5 reveals at least eight protein zones migrating faster than albumin These are in order of decreasing migration rate thyroxine binding prealbumin, orosomucoid, the Pi proteins and three proteins not yet identified The Pi proteins stain like glycoproteins and correspond to α_1 antitrypsin

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POLYOMA VIRUS

3 On the Nature of the Virus Receptors on the Mouse Embryo Cells

By

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Received 6 vi 66

The importance of mucoproteins for the adsorption of polyoma virus to guinea pig red blood cells is well established Treatment of the red blood cells with agents like neuraminidase influenza virus or KIO_4 renders the cells inagglutinable by polyoma virus (*Hartley et al* (9) *Mori et al* (10)) The polyoma virus itself is devoid of receptor destroying activity (*Deinhardt et al* (3) *Hartley et al* (9))

Polyoma virus is adsorbed to both susceptible and insusceptible tissue culture cells (*Crawford* (1), *Dahl et al* (2)), and the virus can subsequently be released by neuraminidase or other agents which indicates that the cell bound receptors are mucoproteins with terminal sialic acid residues The role of the mucoprotein receptors by infection of the cells with polyoma virus was not investigated *Mori et al* (10) found that treatment of mouse embryo cells with neuraminidase had no effect on a subsequent infection with polyoma virus as measured by the production of virus haemagglutinins Contrary to this *Rowe* (11) states that mucoprotein receptors apparently are involved in the entry of polyoma virus into cells since treatment of tissue culture cells with neuraminidase significantly reduced their susceptibility to polyoma virus infection

Stoker & Abel (13) in an attempt to alter the virus receptors on BHK21 cells found that polyoma virus rapidly damaged neuraminidase treated cells This effect was not necessarily due to the neuraminidase itself since their enzyme preparation was impure

In the present report the plaque technique has been used to investigate the effect of neuraminidase on the infection of mouse embryo cells with polyoma virus It was found that the treatment of mouse embryo cells with a purified neuraminidase preparation had a significant effect on the subsequent adsorption of polyoma virus as measured by the number of plaques which developed

It is shown that porcine submaxillary mucoproteins protect the cells against the receptor destroying activity of the enzyme

MATERIALS AND METHODS

Virus A subline[1] of SF polyoma virus was used for all experiments The virus had undergone several passages in monolayer cultures of primary mouse embryo cells The source of virus was infectious tissue culture fluid which had been clarified by low speed centrifugation (10 min at 800 × g)

Cell cultures Primary mouse embryo cells were prepared by trypsinization of minced embryos from mice in the 12th to 14th day of gestation The cells were grown in 1000 ml Roux bottles seeded with 75 × 10^6 cells in 75 ml of medium The cells were grown in Eagle s minimum essential medium (*Eagle* (6)) with Hanks balanced salt solution and a supplement of 20 per cent calf serum After 2 3 days at 37° C a confluent monolayer had formed

Plaque assay Plaque assay was carried out on secondary mouse embryo cell cultures obtained as follows Confluent primary cultures were treated with 0.25 per cent trypsin in Hanks balanced salt solution for 5 10 minutes at 37° C The cells were suspended by gentle agitation After low speed centrifugation the cells were suspended in medium and diluted to 0 5 × 10^6 cells per ml 60 × 15 mm tissue culture plastic dishes (Falcon Plastics) were seeded with 2 5 × 10^6 cells in 5 ml Eagle Earle s medium (Eagle s minimum essential medium with twofold concentrations of amino acids and vitamins in Earle s balanced salt solution supplemented with 0 5 per cent lactalbumine hydrolyzate) with 20 per cent calf serum The cultures were incubated at 37° C in 3 per cent CO_2 in air Complete monolayers had formed after incubation for 24 hours The monolayers were washed once with 2 5 ml of Dulbecco s phosphate buffered saline (*Dulbecco & Vogt* (5)) supplemented with 1 per cent horse serum and Eagle s amino acids and vitamins in twofold concentrations (DPBS) The cells were infected with virus diluted in the same medium The inoculum was removed after adsorption for 2 hours and the monolayers were washed twice with 2 5 ml DPBS All solutions used were equilibrated at the temperatures used for virus adsorption The plates were overlaid with 7 ml of an agar nutrient mixture containing 2 per cent Bacto agar (Difco) and Eagle Earle s medium with 2 per cent horse serum After incubation for 4 days the plates were refed with 3 ml of the above agar nutrient mixture Refeeding was repeated on the 8th and 13th day of incubation The last refeeding contained neutral red to give a final concentration of 1 40 000 The number of plaques was counted on the following day

Neuraminidase Neuraminidase solutions[2] containing 100 or 500 units per ml in sodium acetate acetic acid buffer pH 5 5 were diluted in DPBS or in Eagle Earle s medium to give 5 0 or 0 5 units per ml One unit is defined as the amount of enzyme which splits off 1 µg acetyl neuraminic acid from orosomucoid in 15 minutes at 37° C in the above mentioned buffer After neuraminidase treatment the cultures were washed 3 times with 2 5 ml of DPBS

RESULTS

Effect of Neuraminidase on the Subsequent Infection of the Cells with Virus

The mouse embryo cells were treated with 5 0 or 0 5 units of neuraminidase per ml for 2 or 24 hours at 37° C When the cells were incubated with the enzyme for 24 hours the neuraminidase was added at the time of seeding the plates This prolonged treatment had no effect on cell growth Immediately after the enzyme treatment the monolayers were infected with virus diluted in DPBS The initial cell virus interaction took place at 4° C Table 1 shows that the enzyme

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1 Originally obtained from Professor *Herbert Morgan* University of Rochester and designated SF polyoma virus U S V I

2 Isolated from *Vibrio cholerae* Behringwerke AG The preparation was claimed to be free from detectable amounts of aldolase and proteolytic enzymes

had a pronounced effect on the number of plaques obtained Both con centrations of neuraminidase had effect, although 5 units per ml was required for complete prevention of virus adsorption

TABLE 1

Effect of Pretreatment of Mouse Embryo Cells with Neuraminidase on the Number of Plaques Obtained after an Initial Cell Virus Interaction at 4° C

Pretreatment		Plaques per monolayer			
Units of neura minidase/ml	Hours				Mean
0 5	2*	26	25	18	23
0 5	24**	2	8	10	7
5 0	2*	2	0	0	1
5 0	24**	1	0	0	0
No treatment		34	38	38	37

* Neuraminidase diluted in 2 5 ml DPBS

** Neuraminidase diluted in 5 0 ml Eagle Earle s medium with 20 per cent calf serum

Cell cultures were treated with 5 units for 24 hours, followed by cell virus interaction for 2 hours at 4° C Undiluted virus was used and normally 10–20 per cent of the cells would have been infected By trypan blue staining up to 24 hours after infection no damage due to a combined effect of neuraminidase and virus as described by *Stoker & Abel* for BHK21 cells (13) was found

TABLE 2

Effect of Pretreatment of Mouse Embryo Cell Monolayers with Neuraminidase on the Number of Plaques Obtained after an Initial Cell Virus Interaction at 4° or 37° C

Pretreatment		Plaques per monolayer after cell virus interaction at							
Units of neura minidase/ml*	Hours	4° C				37° C			
					Mean				Mean
5	2	3	3	2	3	0	1	3	1
Diluting medium	2	27	45	46	39	25	17	32	25

Diluted in 2 5 ml DPBS

Table 2 shows that the effect of pretreatment with neuraminidase was the same whether the initial cell virus interaction took place at 4° or 37° C

The Specificity of the Neuraminidase Effect

The neuraminidase was heated at 56 C f r 30 minut s and Table 3 shows that treatment of the cells for · · irs at 37 C with this in

activated enzyme did not remove any receptors necessary for the in fection of the cells with polyoma virus

TABLE 3

Effect of Pretreatment of Mouse Embryo Cell Monolayers with Active and Inactivated Neuraminidase on the Number of Plaques Obtained after an Initial Cell Virus Interaction at 4° C

Pretreatment: Units of neura minidase/ml	Hours	Plaques per monolayer			Mean
5 active	2	4	2	5	4
5 inactivated§	2	165	151	171	162
Diluting medium	2	145	148	114	136

* Diluted in 2 5 ml DPBS
§ Inactivated at 56° C for 30 minutes

Mucoproteins from porcine submaxillary glands[1] were mixed with neur aminidase and the cells were treated with this mixture for 2 hours at 37° C prior to cell virus interaction at 4° C The mucoproteins have a high content of sialic acid in terminal position and protected the cell bound virus receptors against the action of neuraminidase (Table 4) This effect is probably due to competitive inhibition of the neuramini dase receptor reaction

TABLE 4

Effect of Pretreatment of Mouse Embryo Cell Monolayers with a Mixture of Neuraminidase and Porcine Submaxillary Mucoproteins on the Number of Plaques Obtained after an Initial Cell Virus Interaction at 4° C

Pretreatment: Units of neura minidase ml	Pretreatment: mg mucin/ml	Plaques per monolayer			Mean
0 0	0 0	119	132	106	119
0 5	0 0	50	64	64	59
5 0	0 0	6	7	6	6
0 0	2 0	141	135	134	137
0.5	2 0	112	104	102	106
5 0	2 0	11	11	10	11

Diluted in 2 5 ml DPBS

Effect of Post Treatment with Neuraminidase

The initial cell virus interaction was carried out at 4° or 37° C and the cultures were then treated with 5 units per ml of neuraminidase for 2 hours at 37° C Table 5 shows that when the cell virus inter action

[1] Sigma Mucin type III

ook place at 4° C the adsorbed virus was susceptible to the action of he enzyme, whereas by interaction at 37° C part of the virus escaped the effect of the neuraminidase

TABLE 5

Effect of Treatment of Mouse Embryo Cell Monolayers with Neuraminidase after an Initial Cell Virus Interaction at 4° or 37° C

Post treatment		Plaques per monolayer after cell virus interaction at							
Units of neuraminidase/ml	Hours	4° C			Mean	37° C			Mean
5	2	3	0	0	1	13	12	12	12
Diluting medium	2	25	24	31	27	30	30	27	29

* Diluted in 2.5 ml DPBS

DISCUSSION

The present results indicate that sialic acid residues play an important role by the infection of tissue culture cells with *polyoma* virus Treatment of the cells with neuraminidase prior to infection decreased the number of plaques significantly and the fact that mucoproteins with a high content of terminal sialic acid residues protected the cells from the action of the enzyme strongly indicates that the removal of sialic acids is responsible for the decreased yield of plaques A general toxic effect of the neuraminidase preparation on the cells can be ruled out as cells treated with 5 units of enzyme per ml for 24 hours prior to virus infection grew as well as the untreated cells judged from the cell numbers Any killing of the cells as reported by *Stoker & Abel* (13) due to a combined effect of neuraminidase and virus was not found in our experiments

Mori et al (10) found that neuraminidase had no effect on the infection of mouse embryo cells as measured by the production of polyoma virus haemagglutinins Their results however indicate a decreased production of virus in the enzyme treated cells This is due to the fact that neuraminidase which was continuously present after infection releases virus otherwise bound to cells and subcellular fragments (*Crawford* (1) *Hartley & Rowe* (8))

It has been shown for several cell types (*Forrester et al* (7) *Ruhenstroth Bauer et al* (12)) that sialic acid residues derived from mucopolysaccharides and mucoproteins are among the more important ionogenic groupings situated on the cell surface Thus the effect of neuraminidase may not be the removal of virus acceptors per se but altering the structure of the cell surface by splitting off sialic acids in such a way as to make the receptors unable to combine with the polyoma virus

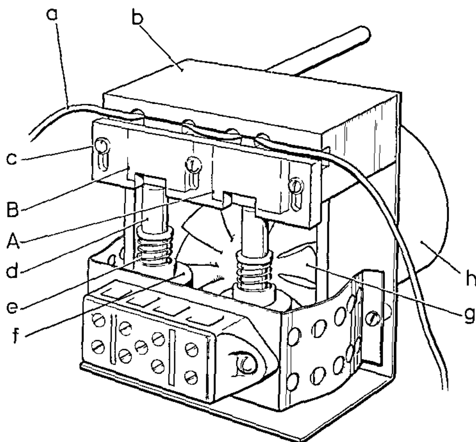


Fig 1

A Narrow valve	B Broad valve
a Rubber tubing	b Plexiglass housing
c Movable lid	d Rod from electromagnet
e Spring	f Electromagnet
g Fan	h Fan motor

DESCRIPTION OF THE ARRANGEMENTS

Technical Details

Magnetic valve device The construction of the magnetic valve device is shown in Fig 1 Part of the feeding line consists of rubber tubing (a) placed in a channel of a plexiglass housing (b) The front of the channel can be opened through a movable lid (c) to enable the insertion of the rubber tubing without disassembling the line The bottom of the channel is partly formed by the cams of the valves A and B which rest on spring loaded rods (d) each extending from a DC electromagnet The springs (e) press the rods against valves A and B closing the rubber tubing when the current to the electromagnets is "off" In "on" position, the electromagnets counteract the springs and unload valves A and B This arrangement also serves as a safety precaution against

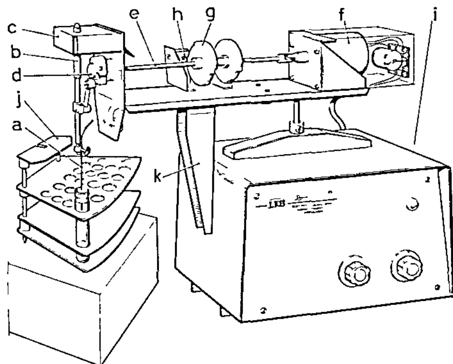


Fig 2

a Needle	b Guide bushing	c Plexiglass block
d Eccenter	e Axis	f Synchronous motor
g Cam	h Microswitch	i Distributor
j Upper plate	k Support over which the distributor arm moves on a roller	

flooding at a failure of the current The small amount of heat generated by the almost constantly working electromagnets is removed by a permanently blowing fan (g)

Needle arrangement The device regulating the upward and downward movements of the needle is shown in Fig 2 The needle (a) is attached to a rod running through a guide bushing (b) fixed in a plexiglass block (c) The rod carrying the needle, as well as the eccenter (d) governing it, are components of a Husqvarna sewingmachine (Husqvarna, Sweden) The eccenter is through an axis (e) connected with a synchronous motor (f) running at 15 rpm The axis carries two cams (g) each acting on a microswitch (h) One of the switches prevents erronous impulses reaching the collector and distributor (i) when the needle is not at its zenith, the other stops the motor when the needle has reached the highest and respectively the lowest position

Operation

The working schedule of the magnetic valves and the movable needle is illustrated in Fig 3 Phase "a" shows the collection of effluent in a

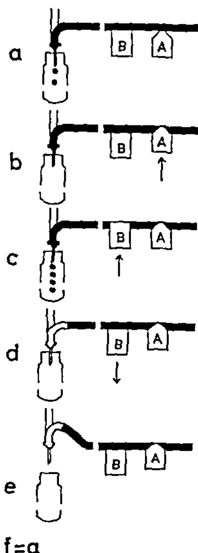


Fig 3

Working schedule of magnetic valves (A and B) and needle device
For explanations see text

tube the membrane of which has been perforated by the needle Valves A and B are open In phase "b valve A has closed the feeding tubing before tube transfer Shortly afterwards valve B (phase "c) has pressed out a small amount of fluid the volume of which is determined by the length of cam B into the tube A moment later when valve B has been released (phase d) an amount of air corresponding to the fluid pressed out has been sucked up into the distal part of the delivery system together with any drop of fluid remaining on the point of the needle The latter is also wiped off by the membrane wh [illegible] ee lle

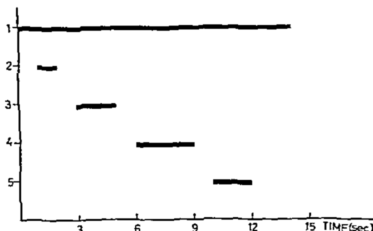


Fig 4
Time scheme for the function of the magnetic valves the movable needle and the tube transfer

1 Closing period of valve A   2 Closing period of valve B
3 Raising movement of needle   4 Period of tube transfer
5 Lowering movement of needle

The time when valves A and B are open (i.e the collection period) can as usual be varied

is drawn up (phase e) to its highest position This phase is followed by a tube transfer after the completion of which the needle is lowered to perforate the membrane of the new tube and to reach again its lowest position Valve A which has been closed since phase "b , will now open to give the same conditions as illustrated in phase "a" A time scheme for the events recorded above is given in Fig 4

Additional Equipment and Precautions

To enable the use of short (110 mm long) screw capped tubes each rack has been supplemented with a short upper plate (Fig 2 j) to be fastened to the rack holder of the rotator In this way the racks can be *lowered sufficiently deeply into the cooling trough* The coil springs of the racks ("tube retainer of LKB) prevent the tubes from being lifted when the needle is drawn up

The mouth of each tube is covered by a foam plastic membrane (autoclavable polyether or polyester thickness 3 mm) overlaid with aluminium foil The membrane is fastened around the tube neck with rubber strings

The racks loaded with tubes are autoclaved wrapped in aluminium foil During fraction collection they are protected by the dust cover of LKB Since the tubes are covered and the [illegible] only by the glass but also by the racks and the [illegible] through ultra

violet light may probably be used under the dust cover without damage to sensitive materials

The devices described above have so far been used for pump-fed columns only. An arrangement with a closed drop counter mounted in the line before the magnetic valves also seems possible, however. The open siphon dispenser on the other hand is of course unsuitable. Actually, such a system must be regarded as a very dangerous one for the collection of infectious material owing to the violent emptying and the formation of bursting bubbles at the delivery tip.

With the arrangements described, contamination of fractions may be avoided provided the fractionation system is sterilized and the only risk remaining for the spread of dangerous material would be that of lip-splashing when the needle is withdrawn from a tube. However, this appears to be minimized as the needle is empty and drawn out slowly without producing any vacuum and, furthermore, the thick membrane has a relatively low elasticity.

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GLUCOSE 2,3,5 TRIPHENYLTETRAZOLIUM CHLORIDE-REDUCTASE IN CELL CULTURE

By

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Received 16 vi 66

A 2 3 5 triphenyltetrazolium chloride reductase (TTC) method serves to distinguish between carcinogenic and non carcinogenic compounds painted on mouse skin (*Iversen & Evensen* 1962) An area of epidermis which had been painted with carcinogens reduces TTC to triphenyl formazan (TPF) at a higher rate than a comparable non painted con trol area on the same mouse No such increase in TTC-reduction has been observed following painting with non carcinogenic compounds

Is is now generally held that there is a direct relationship between the amount of reduced tetrazolium deposited in a cell after proper incubation and the metabolic activity of the same cell (*Iversen* 1959) This view is supported by *Slater et al* (1963), who found that the reduction of TTC in rat liver tissue suspensions is much like the re duction of oxygen and is coupled with cytochrome oxydase

It would be interesting to know if cultured cells infected with onco genic viruses would reveal a similar increase of TTC reduction as mouse epidermis painted with carcinogenic compounds

The problem of applying the TTC-method to cell culture will be discussed in the present paper A following publication is concerned with the results obtained with the TTC-method in tissue cultures in fected with oncogenic viruses

MATERIALS AND METHODS

Mouse embryo fibroblasts (MEF) were prepared by trypsinization of minced em bryos The cells were cultured in Earle Hank [illegible] 10 per cent inacti [illegible] bottles were seede [illegible] 2 or 3 days The [illegible] ization The cells were transferred to 10 ml tubes previously cleaned by soaking for 1 day in a mixture of 600 ml saturated potassium dichromate in water and 1100 ml con centrated sulfuric acid The tubes were then rinsed 3 [illegible] in tap water and 10 times in distilled water

Each tube was seeded with 0 2 mill cells in 1 m [illegible] Earl [illegible] medium supple

The author wishes to thank Research Engineer F [illegible] who has assisted with statistical calculations

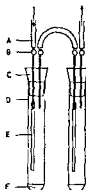


Fig 1

Illustration of the incubation of cell cultures with TTC. Two serially connected tubes. The arrows show the direction of the continuous flow of nitrogen. A Rubber hose. B Hypodermic needle. C Rubber stopper. D Glass tube. E Teflon hose. F TTC incubation medium covering the monolayer

mented with 2.5 per cent inactivated calf serum and 0.3 per cent lactalbuminhydrolysate. The cells were growing on the glass surface at the bottom of the tube. The test tubes were covered by an aluminium sheet and placed in a humidified CO_2 containing atmosphere at 37° C. The pH was 7.3–7.4. Monolayer was obtained within 1–2 days.

Estimating the TPF production, the medium in each culture tube was first replaced by 1 ml buffer (tris/H) which consists of 1 part 0.15 M tris pH 7.4 and 2 parts Hanks' solution without indicator or glucose. After a few minutes this medium was changed to ½ ml 0.01 M TTC in tris/H containing 0.1 per cent glucose. Thereafter the culture tubes were incubated for 1 hour in a water bath at 37° C. Anaerobic conditions were obtained by continuous flow of nitrogen through 3 or 5 serially connected tubes (Fig. 1). The flow was started 5 minutes before incubation and was stopped after 1 hour in the water bath. There should be no bubbling. Immediately after the incubation 2 ml methylcellosolve were added to each tube for extraction of TPF and arresting the tetrazolium reduction (*Hershey et al* 1958). This extraction was usually completed within 1 hour at 20° C. Particles were removed by centrifugation. The content of TPF in the supernatant fluid was estimated in Zeiss spectrophotometer at 490 mμ. By this procedure TPF is stable and the absorption does not change after two days in diffuse day light (*Hershey et al* 1958). When TTC is added to the cultures, however, they should not be exposed to light to exclude a possible source of error.

By adding phenazine methosulfate (PMS) to the TTC incubation medium in concentrations of 10^{-4} to 10^{-6} g/ml the TPF production will increase. This compound is handled in darkness.

RESULTS

The TPF-production in cell culture will increase by incubation with glucose. The production of TPF at different glucose concentrations is illustrated in Fig. 2. There is no further increase in TPF-production at glucose concentrations above 0.02–0.04 per cent.

The TPF-production at different TTC concentrations is illustrated in Fig. 3. At about 10 mM TTC there is no further increase in TPF production. On the contrary, higher concentrations of TTC than seen in Fig. 3 will depress TPF-production. Addition of PMS to the TTC incubation medium will increase TPF-production. Maximum of TPF production is obtained with 10^{-5} g/ml of PMS (Fig. 4).

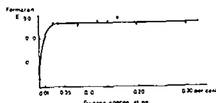


Fig. 2

The effect of glucose concentration on TPF production. Standard procedure using 2 ml tris/H instead of 1 ml before incubation with TTC. Each symbol represents the mean of 3 estimates

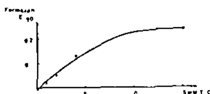


Fig. 3

The effect of TTC concentration on the TPF production. Eac symbol represents the mean of 3 estimates

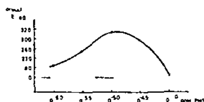


Fig. 4

The effect of PMS on TPF production. Each symbol represents the mean of 3 estimates. The broken line indicates the control level of TPF production without the addition of PMS

The rate of TPF-production is constant for the first 2 hours, with and without addition of PMS. This rate, however, is nearly doubled in the cultures where PMS is added (Fig. 5).

The rate of TPF-production will decrease considerably in the presence of oxygen (*Matsuoka et al.* 1963). There must be 50 to 100 times as many cells by aerobic as by anaerobic conditions to get measurable amounts of TPF. Satisfactory anaerobic conditions is obtained in the first 8 serially connected tubes by continuous flow of nitrogen (Fig. 6).

The effect of pH variation is illustrated in Fig. 7. A minimum of TPF production is found at pH 7.0. From pH [illegible]0 to 7.5 the production increases. At pH 8.0 *there is a plateau.*

The standard deviation for the TPF value obtained for each tube in a separate experiment was approximately [illegible] per cent of the mean. The

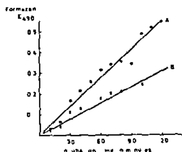


Fig 5

The incubation time in minutes (abscissa) related to the extinctive value (ordinate) Each symbol o or x represents the mean of 5 estimates Curve A Standard TTC method with addition of 10^{-5} g/ml of PMS, curve B Standard TTC method MLF cells

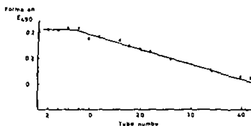


Fig 6

The effect of serially coupling of the continuous nitrogen flow on 42 MFI cultures The ordinate shows the TPF production Each symbol indicates the mean of 2 neighbour culture tubes The abscissa shows the number of the serially coupled tubes The flow of nitrogen starts at the tube number 1

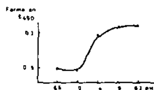


Fig 7

The effect of pH on TPF production The pH of the tris/H and of the TTC incubation medium is indicated (abscissa) The corresponding extinction values are also shown (ordinate) Each symbol represents the mean of 5 estimates

average TPF-production, however, often differed in different experimental series This is probably due to small differences among the series and may depend on cell number, the passage history of the cells, the medium, the temperature, the anerobicity etc These sources of variation and error were largely eliminated by calculating the result in a relative manner using control samples Thus the method was tested in a statistical set-up using 140 and 200 test tubes These 2 groups were investigated at different times and in the same way as a virus-material There were as many "treated groups" as control groups Each such

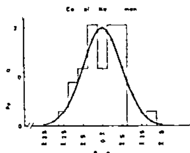


Fig 8

Determination of the standard deviation of the method The ordinate indicates the per cent of quotients The abscissa shows the ratio between the mean of the extinction values of 2 equal groups of MEF culture tubes which both received no treatment Each group consists of 5 tubes 1 2 and 3 standard deviations are indicated The histogram demonstates the observed numbers of quotients in per cent the curve is a normal distribution with corresponding standard deviation

subgroup included 5 tubes In this experiment control groups were identical with the treated groups which received no virus The ratio between TPF production in each treated group and each corresponding control group was calculated The standard deviation of the distribution of the 14 quotients of the first portion of the material is 3 68 per cent and of the 20 quotients of the second portion is 5 70 per cent The standard deviation of the distribution of the total 34 quotients is 5 1 per cent

The distribution of the quotients of the total material did not differ significantly from a normal distribution (χ^2 test P = 0 05 Fig 8) A log normal distribution would be more correct but for ratios near to 1 0 the difference is insignificant

DISCUSSION

The underlying mechanism of the TTC-reaction is not fully understood Several papers however have appeared discussing this matter The reduction of tetrazolium salts has been studied by *Nachlas et al* (1960) *Lester & Smith* (1961) *Slater et al* (1963) TTC is coupled to the cytochrome oxydase in the terminal part of the respiratory chain (*Slater et al* 1963) and the tetrazolium reducing ability and the consumption of oxygen are related (*Laursen & Laursen* 1958 *Hershey et al* 1958) *Kuhn & Jerchel* 1941) who first described the reduction of tetrazolium in biological materials stated that the reaction is not due to glutathion ascorbic acid or cystein which only act at pH higher than 9 0 Reducing sugars are only active at pH above 11 0 (*Hall* *et al* 1947

Electrons are probably transmitted directly from dehydrogenases to tetrazolium by PMS (*Farker & Bueding* 1) and the production of formazan will increase (*Slater* 1963) It has been reported however

that higher concentrations of PMS will suppress the formazan production (*Hashimoto et al* 1964 *Fisher & Bentley* 1964)

The TTC-reaction of mouse epidermis is performed without addition of any substrate and the endogenous substrate is shown not to be metabolized in 1 hour (*Iversen* 1959) So there is reason to assume then that the endogenous substrate in mouse skin is available in sufficient quantities for the TTC reaction In cell culture however the TTC reduction decreases considerably without addition of glucose (Fig 2)

The rate of TPF production is constant at least for the first 2 hours of incubation with TTC and glucose (Fig 5) So it can be concluded that there is no known limiting factor demonstrable by the present method in cell culture which can influence the TTC reduction for the first 2 hours of incubation One hour of incubation with TTC has been used by many authors for instance by examination of epidermis (*Iversen* 1959 *Iversen & Evensen* 1962) and by examination of white blood cells (*Mitsuhashi et al* 1958)

The production of TPF depends upon the concentration of TTC 10 mM TTC is considered to be the lowest concentration which gives the highest and most stable TPF production This concentration was also used by *Slater et al* (1963) by examination of rat liver homogenate TTC is one of the best preparations because of its high ability to penetrate the tissues its suitable redox potential the monochromatic colour of its formazan and its low toxicity (*Iversen* 1959 *Smith & Harris* 1961)

The production of TPF also depends upon the pH This observation is very similar to that with leucocytes (*Mitsuhashi et al* 1958) By incubation of tissue slices *Pearse* (1960) recommended neutral or slightly acid reaction in the incubation mixture In such slices however some degree of cellular and mitochondrial damage is suspected In cell culture such damage is very unlikely Many authors perform TTC incubation at pH 7 4 (*Smith & Harris* 1961 *Slater et al* 1963) It is also the opinion of the author that this pH level will assure a good estimation of the metabolism of the cells at physiological conditions

SUMMARY

A glucose-2 3 5 triphenyltetrazolium chloride-method used in cell culture is described The method in all probability gives an estimate of the metabolic activity of the cells The method is described in detail Values obtained by the described procedure have been tested statistically and the standard deviation equal to approximately 5 per cent have been found

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OMNI SERUM

A Diagnostic Pneumococcus *Serum Reacting with the 82 known types of Pneumococcus*

By

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Received 22.xi.66

An *"Omni serum"* is a pooled, polyvalent purified concentrated Pneumococcus serum giving capsular reactions in a Neufeld test (*Neufeld* 1902) with all the known types of pneumococci.

The latest publication on the serological classification of pneumococci gives the number of types as *81* (*Lund 1962*). *Recently* a new type, 12A, has been isolated reacting with three diagnostic sera: 12, 44 and 46 (Table 1). The details concerning type 12A have not yet been published (*Lund & Munksgaard*).

In order to obtain an omnivalent pneumococcal serum with capsular titres ≧ 8 for all types, it is necessary to use very potent sera. The technique of immunizing rabbits with pneumococci was described by *Lund* 1960.

An *"Omni serum"* is a *pool of equal amounts of nine polyvalent sera* (A to I). Each of these nine sera reacts with 7–11 types, together covering the 82 types (*Lund* 1963). 0.01 per cent merthiolate is added to all sera. The pooled serum is purified and concentrated by salting out with sodium sulphate (*Bjørneboe* 1938). The precipitate is dialysed, lyophilized and redissolved in saline.

An example of this procedure is as follows: 500 ml of the pooled serum was precipitated by a solution containing 260 gram of anhydrous sodium sulphate in 1250 ml of distilled water. After standing for 30 minutes the mixture was centrifuged for 30 minutes in a ½ litre centrifuge at 10.000 r.p.m. (Titan separator using a closed system).

After repeated centrifugation and decanting the precipitate was transferred to cellophane tubing using a little distilled water. The suspension was dialysed for three days against 25 litres of distilled water which was changed twice daily. During this dialysis the globulins absorbed water to a volume of about 600 ml. After filtration through a Seitz EK-S filter the globulin solution was freeze dried ([illegible]) and redissolved in 1.5 ml of physiological saline.

About [illegible] per cent of the proteins present were removed simultaneously with approximately a fourfold concentration of the specific proteins.

The content of merthiolate was decreased by this method and therefore 0.01 per cent merthiolate and 0.02 per cent methylene blue was added to the finished sera. The serum was found to be unimpaired after storage for 18 months at 4° C.

TABLE 1

Danish Type Designations of Pneumococci with the Antigenic Formulas According to Kauffmann and Lund

Type	Antigenic formulas	Type	Antigenic formulas
1	1a	20	20a 20b 7g
2	2a	21	21a
3	3a	22	22a 22b
4	4a	22A	22a 22c
5	5a	23	23a 23b 18b
6A	6a 6b	23A	23a 23c 15c
6B	6a 6c	23B	23a 23b 23d
7	7a 7b	24	24a 24b 24d 7h
7A	7a 7b 7c	24A	24a 24c 24d
7B	7a 7d 7e 7h	24B	24a 24b 24e 7h
7C	7a 7d 7f 7g 7h	25	25a 25b
8	8a	27	27a 27b
9A	9a 9c 9d	28	28a 28b 16b 23d
9L	9a 9b 9c 9f	28A	28a 28c 23d
9N	9a 9b 9e	29	29a 29b 13b
9V	9a 9c 9d 9g	31	31a 20b
10	10a 10b	32	32a 27b
10A	10a 10c 10d	32A	32a 32b 27b
11	11a 11b 11e 11g	33	33a 33b 33d
11A	11a 11c 11d 11e	33A	33a 33b 33d 20b
11B	11a 11b 11f 11g	33B	33a 33c 33d 33f
11C	11a 11b 11c 11d 11f	33C	33a 33c 33e
12	12a 12b 12d	34	34a 34b
12A	12a 12c 12d	35	35a 35b 34b
13	13a 13b	35A	35a 35c 20b
14	14a	35B	35a 35c 29b
15	15a 15b 15c 15f	35C	35a 35c 20b 42a
15A	15a 15c 15d 15g	36	36a 9e
15B	15a 15b 15d 15e 15h	37	37a
15C	15a 15d 15e	38	38a 25b
16	16a 16b 11d	39	39a 10d
17	17a 17b	40	40a 7g 7h
17A	17a 17c	41	41a 41b
18	18a 18b 18c 18f	41A	41a
18A	18a 18b 18d	42	42a 20b 35c
18B	18a 18b 18e 18g	43	43a
18C	18a 18b 18c 18e	44	44a 44b 12b 12d
19	19a 19b 19d	45	45a
19A	19a 19c 19d	46	46a 12c 44b
19B	19a 19c 19e 7h	47	47a 35a 35b
19C	19a 19c 19f 7h	48	48a

The omni serum is intended for quick diagnosis especially in spinal fluids. With this serum it is possible in a few minutes to identify pneumococci directly in the specimen. It is recommended that an examination with a type 3 serum should always be included as the diagnosis of this type presentes certain difficulties. The omni serum has been described previously (*Lund* 1963) but the practical details concerning the preparation of the serum are described in the present article.

The Statens Seruminstitut produces three kinds of diagnostic Pneu-

mococcus sera 1) 46 type or group sera which react with single types or groups numbered 1–48, no 26 and 30 are not used 2) 9 pooled sera (A–I) together covering all 82 types and 3) an omni serum reacting with all 82 types

SUMMARY

A pneumococcal omni serum gives a capsular reaction with all 82 types It is meant for a quick diagnosis directly in the specimen especially in spinal fluids

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BRIEF REPORT

HISTOCHEMICAL DEMONSTRATION OF Δ^5-3β-HYDROXYSTEROID DEHYDROGENASE ACTIVITY IN CULTIVATED GRANULOSA CELLS OF THE PORCINE OVARY

By *S Bergman*, *L Bjersing* and *O Nilsson*

Bjersing & Carstensen (1964) previously have demonstrated Δ^5-3β hydroxysteroid dehydrogenase activity biochemically in isolated granulosa cells from follicles of the porcine ovary However in spite of fairly extensive studies on this enzyme system in frozen sections of the ovary of pigs (*Bjersing* unpublished observations) and other mammalian species (cf *Ferguson* 1965) only negative or weak reactions have been obtained histochemically in follicular granulosa cells except in atretic follicles As Δ^5 3β-hydroxysteroid dehydrogenase activity quite recently has been demonstrated histochemically in cultures of granulosa cells from horse Graafian follicles (*Channing & Short* 1966) we have performed histochemical investigations on cultivated granulosa cells from follicles of the porcine ovary to supplement the biochemical studies

Methods Porcine ovaries were obtained immediately upon slaughter of animals and granulosa cells were isolated as previously described (*Bjersing* 1962) The cells were transferred to centrifuge tubes containing medium composed as described by *Channing & Short* (1966) 30 per cent Parker 199 60 per cent Hanks balanced salt solution and 10 per cent horse serum except that 10 per cent swine serum replaced the horse serum To the medium 100 U of penicillin and 100 γ of streptomycin per ml was added After cultivation in Carrel flasks for a week at 37° C incubation at 37° C for histochemical demonstration of hydroxysteroid dehydrogenase activity was undertaken The incubation medium was composed as shown in Table 1 (modified from *Levy et al* 1959)

TABLE 1

Medium for Visualizing Hydroxysteroid Dehydrogenase

Hydroxysteroid in propylene glycol	(1.3 mg/ml)	0 15 ml
Tetranitro blue tetrazolium	(1 mg/ml)	1 0 ml
Nicotinamide	(1 6 mg/ml)	0 7 ml
NAD	(3 mg/ml)	0 8 ml
Phosphate buffer (Sørensen)	(0 1 M pH 7 4)	4 0 ml

In the controls which were always incubated in parallel the medium contained all the reagents except the hydroxysteroid For demonstrating $NADH_2$ tetrazolium reductase (diaphorase) the reduced instead of the oxidized coenzyme was given and the hydroxysteroid omitted

Comments In positive reactions the cytoplasm shows a diffuse black brown colouring of varying strength In negative reactions the cytoplasm is faintly coloured or colourless The histochemical method for demonstration of the hydroxysteroid [illegible] olium salt into [illegible] ater and lipid [illegible] th the salt and [illegible] $NADH_2$ tetrazolium reductase or diaphorase (*Wattenberg* 1958) It is therefore necessary to know the activity of the diaphorase and that is the reason why this enzyme activity also has been studied

Received 20 v 66 *from the Institute of Clinical [illegible]ology and Institute of Pathology University of Umeå Umeå Sweden*

Fig 1

Positive Δ^5 3β hydroxysteroid dehydrogenase reaction in cultivated granulosa cells of porcine ovarian follicles using the tetrazolium salt tetranitro blue tetrazolium as a final electron acceptor × 440

Results The incubations were carried out for about six hours Fig 1 demonstrates fairly strong Δ^5 3β hydroxysteroid dehydrogenase activity histochemically by a six and a half hour incubation with dehydroepiandrosterone as steroid and NAD as cofactor The controls set up in the medium with propylene glycol but without steroid were devoid of significant colouring

Discussion The reason why Δ^5 3β hydroxysteroid dehydrogenase activity can be clearly demonstrated histochemically in cultivated granulosa cells but not in membrana granulosa in frozen sections of porcine ovarian follicles is obscure The granulosa cells in frozen sections of porcine corpora lutea show a fairly strong Δ^5 3β hydroxysteroid dehydrogenase activity (*Bjersing* 1966) Perhaps the isolation and cultivation of the granulosa cells may induce the same changes in the cells as occur in the rupture of the follicle and the formation of corpus luteum It may also be remarked that the cultivated cells represent intact cells where the processes necessary for visualizing the dehydrogenase activity may go on with greater activity and/or for longer periods of time

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BRIEF REPORT

ENHANCEMENT OF STAPHYLOCOCCAL PATHOGENICITY IN THE PRESENCE OF PENICILLIN

By *H O Hallander, G Laurell* and *G Lofstrom*

In a previous paper (1) it was shown that penicillin G and methicillin *in vitro* stimulated staphylococci to produce a haemolysin which was active mainly on rabbit erythrocytes but also and to a minor extent on human and sheep erythrocytes It was suggested but not definitely proved that this toxin was identical to alpha- [illegible] *et al* (3) [illegible] her studied [illegible] in also pos [illegible] the necrotic [illegible]

Materials and Methods White rabbits weighing about 2 5 kg were injected intracutaneously with 0 2 ml of staphylococcus aureus suspensions (strain 13671) containing $5 \cdot 10^{10}$ $1.25 \cdot 10^{10}$ $3 \cdot 10^{9}$ and $8 \cdot 10^{8}$ bacteria per ml The density of the suspensions was measured in a Beckman colorimeter (green filter) and the corresponding values for the number of bacteria were taken from a standard curve in which cell counts in a Burker chamber were plotted against extinction values The bacteria were grown on solid medium covered with cellophane for 18 hours at 37° and washed three times in phosphate buffered saline before injection

Two series of experiments were undertaken

1 Benzylpenicillin sodium was given intramuscularly immediately after the [illegible] eight The [illegible] *procaine* [illegible] after the injection of bacteria in one dose intramuscularly up to 300000 IU per kg of body weight

The results were recorded after 24 48 and 72 hours

TABLE 1

Skin Reaction to Constant Doses of Intracutaneously Injected Staphylococci on Rabbits Given Different Doses of Benzylpenicillin The Results Are Given after 24 and 72 Hours

Penicillin dose per kg body weight (IU)	Number of staphylococci per ml injected intracutaneously							
	$5 \cdot 10^{10}$		$1.25 \cdot 10^{10}$		$3 \cdot 10^{9}$		$8 \cdot 10^{8}$	
$3 \cdot 10^{6}$	(+)	(+)	0	0	0	0	0	0
$3 \cdot 10^{5}$	+	0	0	0	0	0	0	0
$3 \cdot 10^{4}$	+	++	+	++		(+)	0	0
$3 \cdot 10^{3}$	+	+	+	0	0	0	0	0
0	+	++	(+)	0	0	0	0	0

\+ = erythema and swelling
++ = erythema and swelling + suppuration and necrosis

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Results *Experiment 1* As seen in Table 1 staphylococci injected intracutaneously gave an enhanced skin reaction in rabbits which had repeatedly received 30000 IU benzyl penicillin per kg body of weight When a moderate concentration of staphylococci (1 25 10^{10}–3 10^{9} bacteria/ml) was used there was a marked difference between the skin reactions in rabbits which had received 30000 IU per kg body of weight and the rabbits which had been given either no penicillin or higher doses This observation was not valid when larger quantities of staphylococci (5 10^{11} bacteria/ml) were injected

Experiment 2 When 150000 IU penditan comp per kg body of weight were used formation of abscesses occurred from which staphylococci could be isolated A control rabbit given saline instead of penditan comp just showed the injectionmarks but no infiltration during the observation time after the same doses of staphylococci *These experiments were less extensive The results will be published in detail* later and will then be completed by parallel measurements of the penicillin concentrations in skin and serum

Discussion As penicillin was shown to stimulate the haemolysin production of staphylococci *in vitro* it was of interest to study the effect *in vivo* Thus it was demonstrated that the dermonecrotic effect on rabbit was enhanced when penicillin was given at the same time as the staphylococci

Whether this phenomenon is of importance in man is unclear and depends on *the nature of the released toxin As reported earlier (1) the released toxin* was mainly active on rabbit erythrocytes but a certain effect on human erythrocytes was also noticed The nature of the dermonecrotic toxin or toxins produced will be further studied

Summary It was shown that the dermonecrotic effect of staphylococci was enhanced in the presence of penicillin

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MIGRATION OF CELLS FROM THE THYMUS TO THE SPLEEN IN YOUNG GUINEA PIGS

By

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Received 7.ii.66

The thymus is a well known centre of lymphocyte production, indeed the major lymphopoietic organ in many species (*Metcalf* 1964 (review)). Many authors consider that many thymus lymphocytes are continuously leaving the organ while others are of the opinion that at least the vast majority of the cells formed degenerate *in situ* (*Metcalf* 1964, *Sainte Marie & Leblond* 1964 (reviews)). According to *Kindred's* (1942) calculation 90 per cent of the thymus lymphocytes should leave the thymus. However, his results have been met with rather hard criticism mainly from technical points of view (*Andreasen & Christensen* 1949, *Andreasen* 1959). There is no definite direct evidence so far that thymus cells ever leave the thymus in considerable amounts under normal conditions. This lack of knowledge depends mainly on difficulties in labelling the thymus cells selectively. The "ideal" labelling experiment would consist in labelling only the thymus cells *in vivo* and observing them within the animal that has formed them.

The deoxyribonucleic acid (DNA) of cells can be specifically labelled (*Reichard & Estborn* 1951) with tritiated (H^3) thymidine. This compound is much in current use as a label in studies of cellular kinetics (*Hughes et al.* 1958, *Bertalanffy* 1964 (review)). The uptake of injected thymidine seems to start as soon as it comes in contact with DNA synthesizing cells (*Potter* 1959, *Rubini et al.* 1960). It can therefore be assumed that a certain degree of local labelling would follow local injection of H^3 thymidine. *Nossal & Gorrie* (1964) reported on a method of local labelling with H^3 thymidine in the thymus, either by cannulation of the main thymic artery or by direct diffuse subcapsular infiltration. The authors conclude that "if meticulous precautions are taken to minimize spillage, escape of DNA precursor into the general circulation, as judged by labelling of intestinal epithelium, can be kept at an acceptable level". In their experiments they find evidence that lymphocytes emigrate from the thymus to the mesenteric node and also to the

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spleen This work was done with autoradiography in young guinea pigs and only heavily labelled cells were considered as migrants. During the course of the present investigation another work utilizing autoradiography and young guinea pigs was reported (*Murray & Woods* 1964). H^3 thymidine was injected directly into the thymus parenchyma and thymic vessels were ligated to avoid leakage of H^3 thymidine. The extent of label of duodenal cells was used as an estimate of the success of local labelling. In that work there is evidence of a migration of cells from the thymus principally to the mesenteric lymph nodes and also to the spleen.

In the present investigation we wanted an answer to these questions:

1. Is there a quantitatively measurable transport of labelled DNA from the thymus?
2. To which organ(s) is this transport directed?

MATERIAL AND METHODS

Animals. 20 male guinea pigs weight 275–353 gm at the beginning of the experiment were used for these studies. 7 animals were chosen at random for intraperitoneal (i p) injection and 11 animals for *intra thymus* (i t) injection with H^3 thymidine. 2 animals were not injected and later used for background correction in tritium measurements.

Labelling. The animals were labelled by intrathymic or intraperitoneal injection of 25 or 50 µC of H^3 thymidine (spec. act. 3.0 C/mM Schwartz Laboratories Inc New York U S A) (See Table 1). The intrathymic injections of isotope were made

TABLE 1
Animals and Treatments

Animal number	Route of injection	Dose of H^3 thymidine µC	Organs dissected: Reference organs	Organs dissected: Thym
1	i t	25	+	
		25	+	—
3		25	+	—
4		25	+	
5		50	+	
6		50	+	—
7		50	+	
8		50	+	—
9		50	+	+
10		50	+	+
11		50	+	+
12	i p	25	+	
13	"	50	+	
14	"	50	+	
15	"	50	+	+
16	"	50	+	+
17		50	+	
18	"	50	+	+

i t — *intra thymus* injection
i p — intraperitoneal injection

under local anaesthesia (about 0.5 ml 1 per cent xylocain® – Astra, Sweden). The thymus was exposed according to the description by *Gyllensten* (1953). Each animal received 25 µC of the isotope per lobe in a volume of 0.05 ml, injected slowly directly into the parenchyma of the lobe with a tuberculine syringe with a gauge no. 22 needle (external diameter 0.30 mm). Some animals were injected into one, others into both lobes. The 7 animals chosen for intraperitoneal labelling were sham

consumed a time of 10 min.

Dissection procedure. 48 hours after the administration of isotope the animals were killed with ether and the spleen, the mesenteric lymph nodes, one testis, about 10 cm of jejunum and about the same length of ileum were dissected out. The pieces of intestine were cut up and cleaned in 5 per cent trichloroacetic acid (TCA). In some cases the thymus was also dissected out. All samples were put down in 5 ml of cold 5 per cent TCA and then immediately frozen and stored at —20° C until isolated.

Isolation of DNA and liquid scintillation technique. DNA was extracted by a modified Schneider procedure (*Schneider* 1945) in the following way. The organs were homogenized with a refrigerated Buhler homogenizer for approximately 1 minute in 5 ml 5 per cent trichloroacetic acid. The homogenate was then centrifuged at +4° C at about 2000 g for 10 minutes. The supernatant was discarded and the homogenate washed twice with cold 5 per cent TCA, 5 ml each time. With this procedure the residue contains the nucleic acids, and the discarded supernatant acid soluble compounds (*Schneider* 1945) of which tritiated water is the most important (*Lundin* 1963). The nucleic acids were extracted with 5 per cent TCA in a water bath at +90° C for 30 minutes. The DNA content of the supernatant was measured with Burton's modification (*Burton* 1956) of the diphenylamine reaction (*Dische* 1930, *Seibert* 1940) with a Beckman B spectrophotometer at 600 mµ. DNA from sperm (Nutritional Biochem. Corp., Cleveland, Ohio, USA) was used for the standard reference solutions. It was not necessary to separate ribonucleic acid (RNA) and lipids from DNA as these compounds do not disturb the diphenylamine reaction (*Burton* 1956).

The radioactivity was measured in a liquid scintillation counting system (Packard Tri Carb 314 ex) at +2° C using Bray's solution as scintillator (*Bray* 1960), to which 10 per cent water phase (nucleic acids in 5 per cent TCA) was added. Nucleic acids isolated in the same way from the corresponding organs of the 2 non injected animals were used for background correction. The channels ratio method (*Baillie* 1960) was applied to control quenching. Each sample was divided into two portions and each portion was counted twice. In the determinations of specific activity the average of these four values of each sample was used.

CALCULATIONS

Symbols

Spec. act.	— specific activity
Rel. act.	— relative activity
M_T	— mean of rel. act. of an organ *intra thymus* injected animals
M_P	— mean of rel. act. of an organ intraperitoneally injected animals
ε_{M_T}	— standard error of M_T
ε_{M_P}	— standard error of M_P
M_T/M_P	— mean of rel. act. of an organ *intra thymus* injected animals divided by mean of rel. act. of the same organ [illegible] injected animals
ε_{M_T/M_P}	— standard error of quotient M_T/M_P
Q(T)	— spec. act. of an organ in percentage [illegible] of the same *intra thymus* injected animal

$Q(P)$ = spec act of an organ in percentage of spec act of jejunum of the same intraperitoneally injected animal

$M_{Q(T)}$ = mean of $Q(T)$

$M_{Q(P)}$ = mean of $Q(P)$

$\varepsilon_{M_{Q(T)}}$ = standard error of $M_{Q(T)}$

$\varepsilon_{M_{Q(P)}}$ = standard error of $M_{Q(P)}$

The calculations were performed as follow

1 Spec act expressed as counts/min/mg DNA was calculated for all the samples examined
2 For every animal, the sum of the spec act of certain reference organs namely the spleen, the mesenteric lymph nodes, testis, jejunum and ileum was calculated
3 Rel act is defined as the spec act of a sample in percentage of the sum calculated above The rel act of thymus samples were also calculated after the same kind of correlation of the spec act of the thymus sample and the sum of spec act of the reference organs, as described above

As the quantities of isotope injected must be kept rather small to avoid cell damage from pressure a great advantage of this calculation method is that minute differences in the amount injected do not change the rel act of the organs The pattern of label will be the same for different amounts of isotope a change with a factor within reasonable limits in the spec act of an organ will give a change with the same factor in the spec act of the reference organs and does not change the rel act This is also the reason for the comparability of the animals injected with 25 and 50 μC of H^3 thymidine

4 The quotient between the relative activity for the *intra thymus* and the intraperitoneally labelled animals was counted for different organs
5 The standard error (ε) was then calculated for the quotient M_T/M_P according to the formula

$$\varepsilon_{M_T/M_P} = \frac{1}{M_P}\sqrt{\varepsilon_{M_T}^2 + \varepsilon_{M_P}^2 \frac{M_T^2}{M_P}}$$

6 For each animal $Q(T)$ $Q(P)$ $M_{Q(T)}$ $M_{Q(P)}$ and $\varepsilon_{M_{Q(T)}}$ $\varepsilon_{M_{Q(P)}}$ were calculated (The same values could of course be obtained also from figures of relative values)
7 As the standard deviations of $M_{Q(T)}$ and $M_{Q(P)}$ were of the same magnitude Student's t test was applied to compare the quotients $Q(T)$ and $Q(P)$ by the formula

$$t = \frac{M_{Q(T)} - M_{Q(P)}}{\sqrt{\varepsilon_{M_{Q(T)}}^2 + \varepsilon_{M_{Q(P)}}^2}}$$

RESULTS

The spec act were higher for all organs except the thymus of the intraperitoneally labelled animals than for the *intra thymus* labelled ones (see Table 2) The spec and rel act of the thymus of the *intra thymus* labelled animals was very high compared with that of the intraperitoneally labelled ones, although the quantity of H^3-thymidine which is found in DNA of the thymus is small compared with the quantity injected

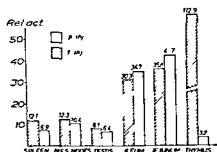


Fig 1

Mean of Rel Act of Different Organs of Intra Thymus (i t) and Intraperitoneally (i p) Labelled Animals

Rel act is defined as the spec act of a sample in percentage of the sum of the spec act of the reference organs, namely the spleen the mesenteric lymph nodes, testis jejunum and ileum

TABLE 2

Mean of Spec Act of Different Organs of Intra Thymus (i t) and Intraperitoneally (i p) Labelled Animals (Values for Animals Labelled with 25 µC Doubled) c/min/mg DNA

Route of injection	Thymus	Spleen	Mes lymph nodes	Testis	Ileum	Jejunum
i t	22.319	1 884	1 859	1.340	4 589	5 576
i p	1 424	2.260	3 124	2 276	10 703	12 049

TABLE 3

Mean and Standard Error of the Mean of Rel Act of Different Organs of Intra Thymus ($M_T \pm \varepsilon_{M_T}$) and Intraperitoneally ($M_P \pm \varepsilon_{M_P}$) Labelled Animals Quotient M_T/M_P and Its Standard Error of the Same Organs of the Same Groups of Animals

Organ	$M_T \pm \varepsilon_{M_T}$	$M_P \pm \varepsilon_{M_P}$	$M_T/M_P \pm \varepsilon_{M_T/M_P}$
Spleen	12 1 ± 0 9	6 9 ± 0 8	1 75 ± 0 24
Mes lymph nodes	12.3 ± 1 0	10 4 ± 0 8	1 19 ± 0 14
Testis	8 1 ± 1 5	6 4 ± 1.3	1 29 ± 0 33
Ileum	30 3 ± 1 7	34 7 ± 2 3	0 87 ± 0 09
Jejunum	35 8 ± 1 2	41 7 ± 3 5	0 86 ± 0 09

M_T/M_P — Mean of rel act of an organ intra thymus injected animals divided by mean of rel act of the same organ intraperitoneally injected animals

The spec act of the samples from the animals labelled with 25 µC H^3-thymidine were lower than that of the samples from animals labelled with 50 µC but the rel act values for the animals labelled intraperitoneally with 25 µC were of the same magnitude as for the animals

labelled intraperitoneally with 50 μC. The same is valid for the animals labelled *intra thymus* with 25 μC, compared with the ones labelled *intra thymus* with 50 μC. Thus, all *intra thymus* labelled animals are included in one group and all intraperitoneally labelled ones in the other.

For jejunum M_T/M_P was 0.86 while it was 1.75 for spleen and 1.19 for the mesenteric lymph nodes. For testis, a rather high M_T/M_P value (1.28) was obtained, but the individual values showed great variation. The quotient M_T/M_P for ileum was about the same as for jejunum, *i.e.* 0.87 (see Table 3 and Fig. 1).

Mean of the figure formed by comparing spec. act. of the spleen with spec. act. of jejunum of the same animal was 34 for the *intra thymus* labelled animals ($M_{Q(T)}$) while it was 18 for the intraperitoneally labelled ones ($M_{Q(P)}$). Student's t test showed a significant ($P < 0.005$) difference for this figure between the *intra thymus* and intraperitoneally labelled animals. The figure formed in the same way between the mesenteric nodes and jejunum was 35 ($M_{Q(T)}$) and 27 ($M_{Q(P)}$) for the *intra thymus* and intraperitoneally labelled animals, respectively. The same figure between testis and jejunum was 23 ($M_{Q(T)}$) and 17 ($M_{Q(P)}$) for the two groups of animals. Between ileum and jejunum it was 86 ($M_{Q(T)}$) and 88 ($M_{Q(P)}$), respectively. A significant difference for the figures was not obtained between *intra thymus* and intraperitoneally animals for other reference organ than the spleen (cf. Tables 3–4).

TABLE 4

Mean and Standard Error of the Mean of Q(T) and Q(P) and P Values of Different Organs for Intra Thymus (i. t.) and Intraperitoneally (i. p.) Labelled Animals

Symbol	$M_{Q(T)} \pm \varepsilon_{M_{Q(T)}}$	$M_{Q(P)} \pm \varepsilon_{M_{Q(P)}}$	P value <
M_Q Spleen/jejunum	34 ± 3	18 ± 3	0.005
M_Q Mes. lymph nodes/jejunum	35 ± 3	27 ± 4	—
M_Q Testis/jejunum	23 ± 4	17 ± 5	—
M_Q Ileum/jejunum	86 ± 8	88 ± 11	

Q(T) — spec. act. of an organ in percentage of spec. act. of jejunum of the same *intra thymus* labelled animal
Q(P) — spec. act. of an organ in percentage of spec. act. of jejunum of the same intraperitoneally labelled animal
$M_{Q(T)}$ — mean of Q(T)
$M_{Q(P)}$ — mean of Q(P)

DISCUSSION

It is highly probable that there has been a certain amount of leakage into the general circulation at every intra thymic injection. The magnitude of this leakage, of course, varies. An intraperitoneal injection can be said to give the pattern of pure leakage, as H^3-thymidine is pass

ing very fast over to the general circulation Thus at an intraperitoneal injection the H^3 thymidine is built into DNA of proliferating cells everywhere in the body and under equal conditions the distribution pattern of label should be about the same from one animal to another

At labelling with no leakage another pattern of label should be expected The thymus should probably still have a high degree of label after 48 hours and the label should be highest in the organ to which thymic cells had emigrated most With the use of a local labelling technique described above a pattern of distribution of label between these two should be expected Leakage should give a pattern as at an intraperitoneal injection and the label from migrating cells can be expected to change that pattern

The higher spec act of all organs except the thymus of the intraperitoneally labelled animals can be explained in different ways The very high spec act of the thymus of *intra thymus* labelled animals is of course part of this explanation but additional mechanisms must also be considered namely a certain labelling of other proliferating tissues in the neck region because of direct leakage out from the thymus and also a transport from the thymus to other organ(s) and/or parts of the organism than the examined ones By forming rel act comparisons can be made of the labelling pattern of *intra thymus* and intraperitoneally labelled animals regardless of the numeric values of spec act for these two groups

Intestinal epithelium is proliferating very rapidly and it should therefore be expected to be labelled as soon as there is leakage As there is no clear evidence of a cell transport from the thymus to jejunum we postulated that jejunum is labelled more by leakage than any other organ examined This should also mean that the lowest quotient M_T/M_P should be obtained for jejunum This is also the case and thus the labelling of jejunum can be considered to be more of leakage origin than that of any other of the organs examined

By forming Q(T) and Q(P) each reference organ is compared with jejunum of the same animal which organ is labelled mainly by leakage Thus a significantly higher $M_{Q(T)}$ than $M_{Q(P)}$ means that thymus DNA or its degradation products has been transported to the organ the spec act of which forms the figure together with jejunum The results of this investigation show that such a transport takes place to the spleen It is also obvious that it is possible to obtain a high local labelling in the thymus

Local labelling of certain lymphoid organs with H^3 thymidine has earlier been successfully performed for the bursa of Fabricius (*Woods & Linna* 1965) and for regional lymph nodes (*Miller* III 1964 *[illegible] & Linna* 1966) The present investigation shows successful local labelling of the thymus and also transport of labelled DNA from the thymus to the spleen This label can be transported mainly on three different levels

as cells;
as DNA and/or its degradation products; and
as thymidine
An export on thymidine level should be expected to be distributed as an intraperitoneal injection of H^3-thymidine, giving a high spec act in the intestine This view is supported by an experiment by *Bryant* (1964) He showed that H^3-thymidine from labelled necrotized liver cells was reutilized to a high extent in the intestine This is however, not the case in this investigation Thus, we find the explanation for our results to be that label is leaving the thymus and "homing" in the spleen on DNA polynucleotide or on cellular level This investigation does not give any information about the mechanism behind "homing" but we find it easiest to consider the participants in such a complex mechanism to be cells and not DNA and/or its degradation products

Certain cell damage at the intra thymic injection, mainly for mechanic reasons, could be expected, in spite of the fact that the treatment of the cells in an experiment with local labelling must be considered as more gentle than in *e g* transfusion experiments These cells could then leave the thymus as dead cells *Diderholm* (1961) made autologous transfusions of *dead H^3-thymidine labelled cells He could* not find any label brought to the organs examined namely spleen, mesenteric lymph node, ileum, liver, lung, skin, peripheral blood and bone marrow Thus, there is no reason to believe that the dead or injured thymus cells should go especially to the spleen A possible explanation for Diderholm's results is that the DNA of dead cells is rapidly broken down and the H^3 would probably be found as tritiated water This way of looking upon the fate of the dead cells is also in accordance with the experiments of *Weisberger et al* (1951) They found that after intravenous injection of P^{32}-labelled dead cells the label was distributed as after an intravenous injection of inorganic P^{32} i e their DNA was very rapidly broken down Thus, the label is probably transported with living cells

The H^3-thymidine, which is injected *intra thymus*, can also be expected to exert some effects One is the possible mitosis stimulating effect of the thymidine, described by *Greulich et al* (1961) This concept has however been heavily criticized by *Baserga & Kisieleski* (1962) Regardless of this effect, we find no reason to believe that thymidine should stimulate the cells to leave the thymus

In spite of the relatively low dosage and the fact that very little of the H^3 injected seems to stay within the thymus DNA, the administration of the isotope directly into the thymus parenchyma could cause arrest of cellular growth and cell damage (*Baserga & Kisieleski* 1962) This should, however be more important in long-time experiments, but it can not be completely excluded This mechanism might decrease the normal flow of cells from the thymus

There remains a possibility that the introduction of H^3-thymidine

directly into the thymus parenchyma causes the cells to behave in a non physiological manner This possibility can not be excluded but it must be pointed out that the experiments with intra thymic labelling are the most physiological ones hitherto made With this method we can obtain information about the total amount of DNA transported from the thymus but not about the degree of labelling of each cell nor about the way in which the cells have been labelled Thus in contrast to auto radiography the DNA transported with weakly labelled cells which appear after mitosis of heavier labelled ones or possibly after reutili zation of label within the thymus is not omitted

As far as we know from the quantitative point of view a certain transport of cells from the thymus to the spleen has not been shown previously A cell transport from the thymus to the spleen (*Fichtelius* 1953 *Fichtelius & Diderholm* 1959 *Fichtelius et al* 1960) and to the lymph nodes (*Harris & Ford* 1964) has been suggested The hypothesis of migration of cells from the thymus received also a strong support from the findings of *Ernstrom* and collaborators (1965) who found an increased content of lymphocytes in the blood leaving the thymus com pared with afferent thymus blood The autoradiographic works done with intra thymic labelling technique also support this view (*Nossal & Gorrie* 1964 *Murray & Woods* 1964) These authors show that a cell transport probably takes place from the thymus to the mesenteric node and the spleen These authors find less label going to the spleen than to the mesenteric node This differs from our findings as no level of significance is obtained for the transport to mesenteric lymph nodes only for the transport to the spleen This can partly be explained by possible different experimental conditions However the advantages of liquid scintillation counting technique to autoradiography are evi dent in quantitative studies (*Lidén & Linna* 1965) the efficiency of autoradiography being about 0 5 per cent (*Baserga & Kisieleski* 1962) and that of liquid scintillation counting utilizing Bray s solution 11 7 per cent (*Bray* 1960) As we have used nucleic acids in TCA in this investigation the efficiency is decreased to about 8 per cent because of quenching

Another possibility why results are different is that many of the cells going to the spleen might not be sufficiently strongly labelled to be distinguishable in autoradiographic work from cells labelled by leakage of H^3 thymidine or from background of other causes but they are included in this quantitative study

Our experiments give no information about the ultimate fate of these migrating cells There are two main possibilities to be considered the cells can become a part of the cellular population or the labelled DNA and other cell constituents can be reutilized in the target organ The cells can also be transported further to other lymphoid organs or parts of the organism but we do not have any information about this transport

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as DNA and/or its degradation products, and
as thymidine

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MEDULLARY CARCINOMA OF THE HUMAN THYROID GLAND

Autoradiographic Localization of Radioiodine

By

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Received 6 xi 66

The so called medullary thyroid carcinoma was defined in 1959 by *Hazard Hawk & Crile* (6) in a study of 21 cases The authors emphasized the diagnostic significance of the presence of amyloid substance in the stroma Some investigators (1 5 10) have suggested that the medullary type of thyroid carcinoma should be placed in the same category as the papillary and follicular carcinoma and thus should be considered a true thyroid tumour derived from follicular epithelial cells It has been claimed that the amyloid substance in the medullary tumour and in its metastases is probably a local product secreted by the neoplastic thyroid epithelial cells *Albores Saavedra et al* (1) studied the amyloid substance of medullary carcinoma by histological histochemical and electron microscopic methods and concluded that the amyloid is produced by the tumour cells and chiefly composed of glucoproteins and acid mucopolysaccharides which may be related to thyroglobulin

The question whether amyloid in medullary carcinoma contains iodine or not seems to be of considerable significance in the discussion of the histogenesis of the tumour The autoradiographic technique *should be suitable in answering this question and the result of such an* investigation is reported below

METHODS

Four cases of medullary carcinoma of the thyroid have been investigated The patients were given radio odine per os 24 hours preoperatively Case No 1 was given ^{125}I and cases No 2–4 ^{131}I

Tissues from the tumours with surrounding normal thyroid parenchyma were *fixed in 10 per cent neutral formaline solution* and embedded *in paraffin* Th autoradiographs were prepared from sections of 5 µ thickness and placed on glas slides prepared with glycerine albumin After drying in air at 37° C they were de paraffinized and again allowed to dry in the same way during 24 hours In dark room the sections were covered with a photographic emulsion (Nuclear Research

h 0 The glasses

side

er an

exposure for 2–16 days the preparations were developed, fixed and stained in haematoxylin. As a control for nonspecific darkening the same procedure was carried out using nontoxic, benign nodular goitre without any content of radioiodine. Though iodine in some forms might be removed by the liquids used, organically bound (radio-)iodine however is considered to remain in situ in fixed tissues.

MATERIAL

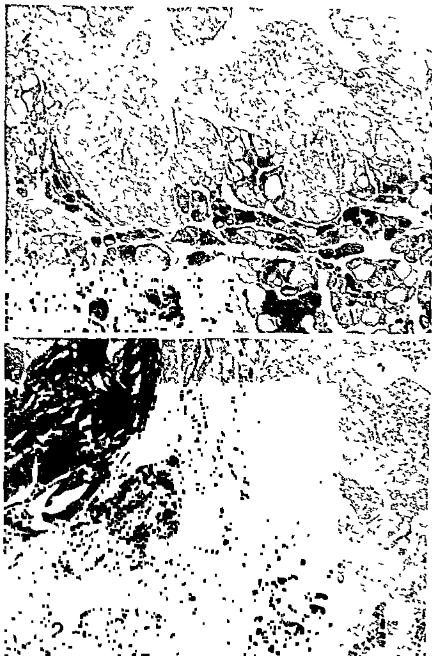
The cases investigated belong to a family with accumulation of medullary carcinoma of the thyroid, pheochromocytoma and multiple cutaneous tumours, which is reported elsewhere (8). Relevant data about the cases are given in Table 1. In all four cases histological examination showed typical medullary carcinoma of the thyroid with amyloid substance in the stroma, which could be demonstrated by the conventional staining methods.

TABLE 1

Case	Sex	Age	Metastases	Thyroidectomy	Dose and kind of radio I given 24 hours before operation
1	♀	43	Tracheal + supraclavicular lymph node involvement	Total + partial tracheal resection	460 µC ^{125}I
2	♀	53	None	Total	1500 µC ^{131}I
3	♀	51	None	Total	1000 µC ^{131}I
4	♂	48	None	Total	500 µC ^{131}I

RESULTS

Best results were achieved in case No. 1 (given ^{125}I). Already after exposure for 2 days a distinct darkening over colloid of the follicles in the normal thyroid parenchyma was seen. Over the tumours, however, no precipitation of silver granules was noticed, neither over tumour cells nor over the amyloid in the stroma (Fig. 1). After exposure for 9 days a slight diffuse precipitation of silver granules appeared over the tumour; this was considered to be caused by exogenous factors, as the control glasses showed the same degree of darkening. Sections from a small follicular adenoma situated close to an area of medullary tumour showed a darkening over the adenoma, which was much more prominent than over surrounding normal parenchyma; the medullary tumour showed no precipitation at all (Fig. 2). Autoradiographs with ^{131}I in cases No. 2–4 gave principally the same results. They showed, however, a considerable degree of blurring because of the relatively bad resolution produced by the high energy β-like radiation of the ^{131}I isotope.



Figs 1 2

Fig 1 Autoradiograph of ^{125}I in medullary carcinoma of the thyroid Case No 1 Above tumour tissue, below normal thyroid parenchyma Distinct darkening over colloid of follicles in the normal thyroid tissue, while no silver pre- [illegible] the stroma of

Fig 2 [illegible] ular ade- [illegible] the right [illegible] 16 days

DISCUSSION

autoradiographic investigation showed no darkening over the our cells, nor over the amyloid substance in the stroma, a finding king strongly against the opinion that the amyloid in the tumour ains thyroglobulinlike, iodinated substances In accordance with finding is the fact, that amyloid in colloid containing papillary or cular carcinoma has not been encountered

he familial disease ' medullary carcinoma of the thyroid associated pheochromocytoma and other neural tumours ' has a multifocal acter, that may suggest a generalized disorder, probably in chromaffin cell system Other investigations which will be re ed have shown that the tumour cells of medullary carcinoma as as those of the phechromocytoma in these patients have certain hemical properties in common which support this idea It has ntly been proposed that the medullary carcinoma may possibly be ved from the so called parafollicular or interstitial cells of the oid (11) These cells have been demonstrated in certain mammalian ies but hitherto not in the human thyroid gland (3)

SUMMARY

familial type of medullary carcinoma of the thyroid has been estigated by an autoradiographic technique, in one case with ^{125}I hree with ^{131}I No organically bound radioiodine could be demon ated over the amyloid substance or the tumour cells It is therefore sidered improbable that amyloid in the stroma of this tumour con is thyroglobulinlike, iodinated substances The character of the ilial syndrome medullary thyroid carcinoma with pheochromo- oma and other neural tumours as a systematic disease probably in chromaffin cell system is emphasized

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GROWTH OF AUTOLOGOUS PERITONEAL FLUID CELLS IN INTRAPERITONEAL DIFFUSION CHAMBERS IN RATS

1 A *Light Microscopical Study*

By

GUNNAR ESKELAND

Received 23 v 66

Peritoneal fluid cells cultured *in vitro* have been observed to form epithelium like cells (1, 2), mesothelium like cells (1), and syncytia (2) Cells of the peritoneal fluid have also been cultured *in vivo* in intraperitoneal diffusion chambers (3 11, 12) The cells used in these experiments were pooled samples implanted in homologous or heterologous hosts and it is not known to what degree immunological incompatibility may have influenced the growth and differentiation of the implanted cells

Observations made in previous work suggested that peritoneal macrophages are capable of transforming to mesothelial cells in peritoneal wounds (4 5) and on the outer surface of intraperitoneal diffusion chambers (6) However, these experiments did not completely exclude the possibility that mesothelial cells might be detached from the peritoneal membrane and settle on the surface of the wounds or chambers

The present investigation was undertaken to study the development of autologous peritoneal fluid cells cultured in diffusion chambers with the express purpose of testing the possibility that cells present in normal peritoneal fluid may transform to mesothelial cells

MATERIALS AND METHODS

Fifty adult male rats 4 6 months old and weighing 200–300 g were operated on under anaesthesia with ether alcohol (2 1) A midline incision was made through the abdominal skin and the muscle and peritoneum were opened by cauter to minimize bleeding

Diffusion chambers (Fig 1 A) were made from acrylic rings to which were glued Millipore HA filter membranes[1] pore size 0 45 μ and thickness 150 μ The rings were

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This investigation was supported by grants from [illegible] Alexander Malthes Legat and from *The Norwegian Research Council for Science and the Humanities*

[1] Millipore Filter Corporation Bedford Mass

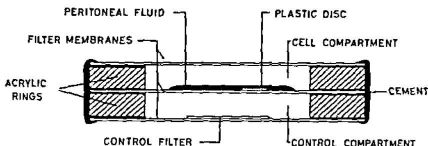


Fig 1 A

Construction of the diffusion chamber The two halves of the chamber were made before the experiment The control compartment was covered by a filter membrane on each side, the cell compartment on one side only Peritoneal fluid was deposited on the upper membrane of the control compartment and the two halves were then united A final seal of cement covering the edges of the chamber was found to be important (see text)

approximately 2 mm thick and had an outer diameter of 32 mm and an inner diameter of 24 mm MF cement (Formulation 1)[1] was used to seal the membranes to the rings

Each chamber was made from two rings and three filter membranes and the assembled chamber thus comprised two compartments the cell compartment and the control compartment separated by the central filter membrane

The two halves of the chamber were made some days before the experiment The control compartment was closed by a filter membrane on each side and contained only a piece of filter membrane (the control filter) which served to check that the chamber was impervious to cells A plastic disc 4 mm in diameter, was punched out of a thin sheet of Vestopal W[2] and placed on the upper filter membrane of the control compartment In the assembled chamber this membrane formed the floor of the cell compartment (Fig 1 A)

The cell compartment was prefabricated with a filter membrane on one side only Before use the two halves of the chamber were stored in an incubator at 78° C for at least 48 hours The filter membranes were moistened with a few drops of saline containing 20 000 I U of penicillin and 0 05 g streptomycin per ml shortly before the peritoneal fluid was collected

Peritoneal fluid cells Peritoneal fluid was collected by means of a capillary pipette the tip of which had been blunted in a gas flame When the tip of the pipette was inserted in the opened abdomen between a coil of small intestine and the parietal peritoneum the narrow part of the pipette filled with peritoneal fluid by capillary force The fluid was deposited on the upper filter of the control compartment and the procedure was repeated until peritoneal fluid was no more readily

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Figs 1–8

Sections of cells grown on the central filter membrane of diffusion chambers

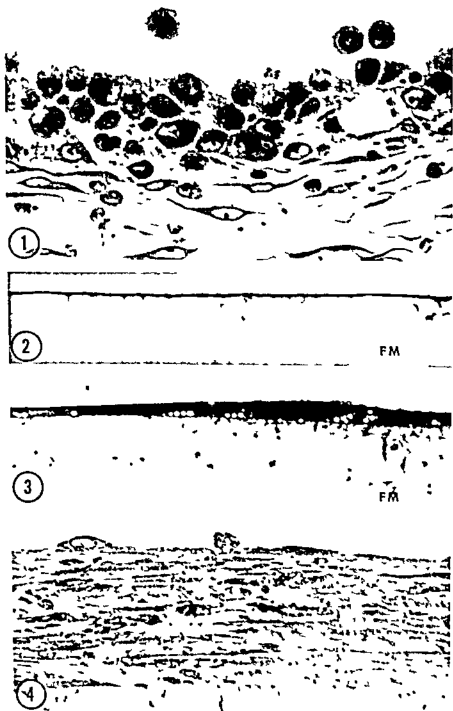
Fig 1 Cells with the appearance of macrophages (top) and fibroblasts (bottom) in a chamber at 3 weeks × 800

Figs 2 3 In some chambers the filter membrane (FM) was covered by a single layer of extremely thin cells in which neither cell junctions nor cytologic details were discernible Cytoplasmic processes extended into the pores of the filter membrane 3 weeks × 800

Fig 4 At 6 weeks several layers of flat cells were usually found, varying amounts of fibrous material and cell debris were present in the intercellular space × 600

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[2] Chemische Werke Hüls AG Marl Recklinghausen W Germany


1
2
FM
3
FM
4

available In this way it was possible to collect 50–150 cmm peritoneal fluid estimated by weighing an equal amount of water Taking the average cell concentration to be 139 000 cells/cmm fluid (4) each cell compartment was charged with approximately 7 21 million cells

The two halves of the chamber were then glued together with MF cement[1] and a generous seal of Tensol cement No 6[3] was applied around the edges of the chamber Pilot experiments had revealed that this extra seal was important for two reasons It reduced the risk of leakage and it covered any rough edges of the rings or membranes which might otherwise invite to adhesion formation

While the cement was setting another drop of peritoneal fluid was collected and used for a smear on a glass slide

The chamber was then placed in the abdominal cavity of the donor rat and the incision was closed in two layers with silk sutures

The chambers were removed after 2 4 6 8 10 or 14 days and then weekly up to 6 weeks after the implantation

Histological procedures After the chamber had been removed and gently blotted with absorbent paper the control compartment was opened by cutting along the inner edge of the acrylic ring The control filter was removed and fixed in 96 per cent alcohol for 1 2 hours Thereupon the cell compartment was opened The plastic disc was removed briefly rinsed in 5 per cent glucose and fixed in ice cold 1 per cent osmium tetroxide in 0 1 M phosphate buffer at pH 7 3 for 1–2 hours A small piece of the central filter was then cut out rinsed in 5 per cent glucose and fixed in either 1 per cent osmium tetroxide for 1 2 hours or in 2 5 per cent glutaraldehyde in 0 1 M phosphate buffer at pH 7 3 for 2 hours followed by osmium tetroxide for 1 hour *The specimens were dehydrated in graded acetones and embedded in Vestopal W* in such a way that sections could be cut in a direction vertical to the surface of the membrane or plastic disc

Sections were cut approximately 1 μ thick on a Huxley microtome They were transferred to glass slides and stained with 0 1 per cent alkaline toluidine blue for 30–40 seconds at approximately 80° C

The remaining part of the central filter was rinsed in glucose immersed in 0 25 per cent silver nitrate solution for 30 seconds and fixed in 96 per cent alcohol for 1 2 hours In some cases the silver nitrate treatment was omitted

These specimens as well as the control filters were stained with hematoxylin or *hematoxylin and eosin dehydrated in graded alcohols and absolute n propanol and* mounted as whole mounts When conventional mounting media were used many preparations turned opaque after some time Cedar oil with a heavy seal of Xam neutral medium[4] along the edges of the coverslip yielded permanent preparations (8)

Smears of the peritoneal fluid were fixed in equal parts of ether and 96 per cent alcohol for 1 2 hours and stained by Papanicolaou s method In a few cases smears were also made from the fluid present in the cell compartment of the chamber and fixed and stained in the same way as the peritoneal fluid smears

RESULTS

Macroscopical Observations

At re operation 41 chambers were found completely free in the peritoneal cavity In 7 animals the omentum or the peritesticular fat had

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Figs 5–8

The filter membranes were covered by a tissue composed chiefly of fibroblast like cells with very long and thin cytoplasmic extensions In some areas the surface cells were round and macrophage like (Fig 6) In other areas the cells on the surface were flat and formed a more or less continuous layer resembling mesothelium (Figs 7 and 8) 3 weeks Figs 5 and 8 × 600 Figs 6 and 7 × 800

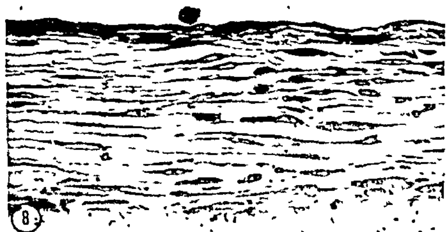
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3 Imperial Chemical Industries Ltd Welwyn Garden City Herts England
4 George T Gurr Ltd London England




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formed adhesions to the edge of the chamber, invariably at a site where the outer seal of cement did not completely cover the rough edge 2 chambers which had perforated the colon and were encased in an inflammatory mass were discarded Chambers which had been implanted for 8 days or more were covered by a discrete tissue capsule, which tended to be thicker with longer implantation periods

The cell compartments of the chambers were filled partly by a clear fluid resembling serum, and partly by a gelatinous clot, the amount of which varied from one chamber to another The control compartments were filled by a similar fluid with little or no gelatinous material

Microscopical Observations

1 *Peritoneal Fluid*

Smears of the free peritoneal fluid showed chiefly macrophages and lymphocytes, a varying number of eosinophil granulocytes and some mast cells (4) Smears with an appreciable admixture of red blood cells also revealed a few polymorphonuclear granulocytes Identifiable mesothelial cells were not observed in any smear

2 *Chamber Fluid*

Smears of the fluid present in the cell compartments of the chambers revealed very few cells They were invariably small with an intensely staining round or oval nucleus and scanty cytoplasm and resembled small lymphocytes

3 *Control Filters*

The filter membranes from the control compartments were devoid of cells but revealed a varying amount of fibrous or amorphous basophilic material

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Figs 9–21

Whole mount preparations of filter membranes

Fig 9 Cells of this appearance were found in practically all the chambers after 8 days of implantation × 800

Figs 10 11 In most chambers after 8 days cells with large pale staining nuclei prominent nucleoli and ill-defined cell borders were found 3 weeks × 900

Fig 12 Mitoses were observed throughout the observation period of 6 weeks × 600

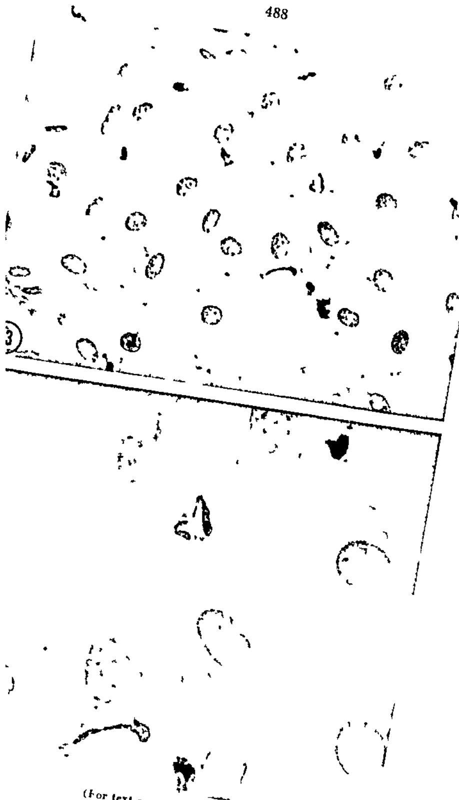
Figs 13 14 (see page 488)

In many chambers especially after 1 3 weeks areas were found in which the appearance and growth pattern of the cells were strikingly similar to those of a simple squamous epithelium Fig 13 × 300 Fig 14 × 800

Figs 15 18 (see page 489)

Figs 15–16 Giant nuclei of cells from chambers at 6 weeks The long diameter of the nucleus in Fig 16 measures approximately 35 μ × 900

Figs 17 18 Multinucleated giant cells in chambers at 3 weeks × 1200



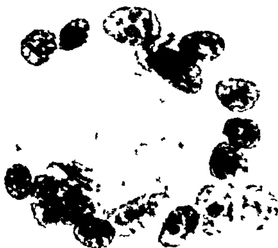
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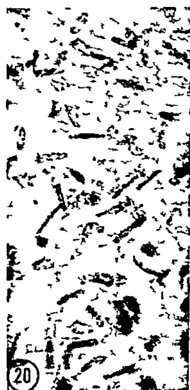


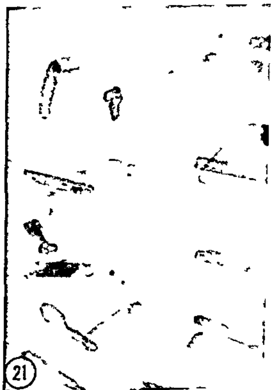
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18

(F r t xt


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21

4 *The Plastic Discs*

The growth of cells on the plastic disc in the cell compartment was generally poor compared to the growth on the adjacent filter membrane The majority of cells were found near the edges of the discs and were extremely flattened These cells are illustrated in a following paper and will not be the subject of further comment here

5 *Cells Growing on the Central Filter Membrane*

a *General remarks* Good growth was found in 37 chambers, poor growth in 7 chambers and no growth, *i e*, many dead cells and no signs of proliferation in 4 chambers—all of which had been implanted for 6 days or less

Many cells seemed to have died in all chambers during the first 2–4 days after the implantation Numerous ghost cells were seen in whole mount preparations of filter membrane from the early chambers The viable cells were of the same appearance as those seen in peritoneal fluid smears In the later stages, few ghost cells were seen

Mitoses were first seen in 4-day chambers and were later numerous in most chambers throughout the observation period

Some of the cell types originally present in the inoculum later disappeared Polymorphonuclear granulocytes, present in small numbers in some of the early chambers, were not observed after the 6th day Eosinophil granulocytes and mast cells gradually disappeared and were rarely seen after 2 weeks Some lymphocytes and macrophages were recognizable throughout the observation period, although the number of unaltered cells tended to decrease with time

b *Sections of the central filter membranes* Fig 1 illustrates a section in which the cells close to the filter membrane were fibroblast like with an abundant intercellular substance, while the cells at some distance from the membrane were macrophages with distinct nucleoli and a more or less vacuolated cytoplasm Other areas displayed a completely different picture (Figs 2 and 3) with the filter membranes being covered by an extremely thin and densely staining layer Neither nuclei nor cell borders could be discerned but numerous vacuoles were some times seen Electron microscopy of adjacent thin sections revealed that this layer was made up of extremely long and thin cells possessing many nuclei without intervening cell junctions (7)

Figs 19–21

Fig 19 In some chambers small cells with deeply staining nuclei contained dark particles in the cytoplasm whereas no such granules were observed in the large cells of the same area 4 weeks × 800

Figs 20 21 At 6 weeks most of the filter membranes were covered with several layers of cells and fibrous material the n[illegible] were u[illegible]al [illegible] oval or rod shaped Fig 20 × 320 Fig 21 × 8[illegible]

In some chambers densely staining particles and masses, probably representing cell debris, were seen intra- and extracellularly (Fig 4)

Areas with several layers of cells resembling fibroblasts were a common finding in most chambers after the first week The cells in the surface layer did not usually show any definite organization (Fig 5) In some areas, however, the superficially located cells formed a more or less continuous layer closely resembling mesothelium (Fig 6, cells to the right, Figs 7 and 8) The underlying cells were fibroblast like with extremely long cytoplasmic processes

c *Whole mount preparations* of the central filter membranes gave a far better survey of the cell population than the sections

After the shorter periods of implantation the cells in most areas were growing chiefly in a single layer (Figs 9–12) Some cells had densely staining, oval or kidney shaped nuclei and a scanty cytoplasm with short tenuous processes (Fig 9) They appeared similar to cells illustrated by *Shelton & Rice* (12) and considered to be lymphocytes The size and stainability suggested that these cells were derived from lymphocytes

Small cells with dense nuclei were frequently seen together with large cells with ill-defined borders and large oval nuclei and prominent nucleoli (Fig 10) Cells of the latter type were also observed growing in sheets without admixture of other cell types (Fig 11), and were found in most chambers implanted for 8 days to 4 weeks Their nuclei resembled the nuclei of fibroblasts cultured *in vitro* and they were probably derived from macrophages

In some areas the majority of cells were small and not easily classified, like the ones illustrated in Fig 12 Mitoses were frequent in cells of this appearance

In other areas the general appearance of the cells and their growth pattern showed a striking resemblance to those of a simple squamous epithelium (Figs 13–14)

Extraordinarily large nuclei were seen in some cells (Figs 15 and 16), compared with the "normal" nuclei in the vicinity

Multinucleated giant cells with up to 100 or more nuclei were found in 24 chambers, sometimes in considerable numbers (Figs 17 and 18) *The nuclei were most often arranged in a circle or in concentric circles* occupying the peripheral parts of the cytoplasm, sometimes they were

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Figs 22–24 26 and 28–32

Whole mount preparations of filter membranes treated with silver nitrate solution before fixation

Fig 22 At 8 days, plaques of cells were observed in which the central cells were smaller and had more deeply staining nuclei than the peripherally located cells × 400

Fig 23 From the edge of a cell sheet like that depicted in Fig 22 × 800

22

23


24


25


26


27

clustered in the central part of the cell Mitoses were never observed in giant cells

In some instances the cells appeared to grow in two layers made up from different cell types (Fig 19) The deep layer consisted of small, stellate or spindle shaped cells with dense nuclei and with prominent granules or particles in their cytoplasm, while the superficial layer was composed of the large cells previously described The significance of the cytoplasmic inclusions in the small cells was not evident but they probably indicate a functional difference between the two cell types

In the older chambers several layers of cells formed a connective tissue with oval or rod-shaped nuclei oriented in crossing directions and with more or less conspicuous parallel fibres between the cells (Figs 20 and 21)

When the filter membranes were treated with silver nitrate before fixation and staining, the cell boundaries were usually clearly visualized In most areas the cells were separated by spaces of varying size In other places collections of large polygonal cells, more or less closely associated, were seen A narrow silver line indicated the borderline between the cells along the whole or a greater part of the circumference Cells of this appearance, together with cells of the type depicted in Figs 13–14, are morphologically clearly different from fibroblasts and will be referred to as epithelium like cells in the discussion

In the early chambers colonies of cells were seen in which the central cells were smaller and had darker nuclei than those at the periphery, probably because the peripheral cells had flattened out (Figs 22 and 23) The large cells had oval, pale nuclei with two or more prominent nucleoli

In many areas the picture resembled that seen on the surface of

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Figs 24–27

Figs 24 and 26 Cells grown in diffusion chambers sometimes presented a picture resembling that seen in regenerating peritoneal wounds From chambers at 8 days

Figs 25 and 27 Micrographs of Häutchen preparations (4) showing surface cells in regenerating peritoneal wounds at 6 days Figs 24 and 25 × 400 Figs 26 and 27 × 800

Figs 28 29 (see page 496)

At 2 weeks sheets of large cells were found frequently growing as an incomplete surface layer on top of the smaller cells Mitoses were seen arrows Fig 28 × 320, Fig 29 × 800

Figs 30 32 (see page 59

Figs 30–31 At 3 weeks plaques of even larger cell [illegible] n at the 2 weeks stage were found Some of these cells measured mor [illegible] n 125 [illegible] 65 µ Fig 30 × 320, Fig 31 × 640

Fig 32 Mitoses in an area similar to that de[illegible] × 1 400

(For text see page 495)







For ex se pag 49

regenerating peritoneal wounds Figs 24 and 26 are illustrations of cells growing on filter membranes, and Figs 25 and 27 are micrographs of Häutchen preparations from peritoneal wounds (4) for comparison

At 2 and 3 weeks, many cells of this type had become very large (Figs 28–31) The silver lines between the cells were frequently incomplete, giving the impression of cytoplasmic continuity or of extremely large cells with several nuclei

Mitoses were frequently seen in all the cell types illustrated, except in the multinucleated giant cells (Figs 12, 24, 28, 29, 32)

DISCUSSION

A remarkable finding in this investigation was the large variability in cell morphology and relationships, not only in different chambers implanted for the same period but also in different areas within the same chamber Furthermore, it was not possible to trace a definite sequence in the morphological changes of the implanted cells

Of the cells originally present in the inoculum, the eosinophils and the mast cells gradually disappeared, while some lymphocytes and macrophages were found to be apparently little changed throughout the observation period

Shelton & Rice (12) observed an abundance of plasma cells in similar diffusion chambers In the light of present day knowledge it seems likely that the plasma cells had formed in response to antigenic stimulation, since the peritoneal fluid cells were pooled samples implanted in homologous or heterologous hosts In the present study very few plasma cells were observed

Shelton & Rice also found foci of granulocytopoiesis, with an extraordinarily high mitotic activity No evidence has been found in this study indicating a new formation of either eosinophilic or polymorphonuclear granulocytes

Apart from the cells mentioned, three fairly well defined cell types were found Fibroblasts multinucleated giant cells, and epithelium like cells In addition, scattered cells were seen which could not be classified with certainty

Fibroblasts, identified on morphological grounds, were found in increasing numbers after the first week of implantation In the older chambers fibrous material was discernible between the cells Proof that these cells were fibroblasts *i e* collagen-producing cells, will be presented in a succeeding paper (7) The most likely source of the fibroblasts were the peritoneal macrophages, and some cells were observed which might conceivably represent intermediate stages between macrophages and fibroblasts *Shelton & Rice* (12) found reason to believe that lymphocytes might also modulate into fibroblasts The present study has not provided evidence to support such a transformation of lymphocytes

Multinucleated giant cells of the type illustrated in Figs. 17 and 18 were commonly found. The origin of this type of cells and their mode of formation have been discussed in several papers. *Lewis* (10) considered that they arose from blood monocytes either by division of nuclei without division of cytoplasm or by the fusion of cells. *Gillman & Wright* (9) found that cells of this type formed *in vivo* by the fusion of lymphocytes or monocytes. *Sutton & Weiss* (13) observed that monocytes *in vitro* transformed to epithelioid cells which by fusion formed multinucleated giant cells. Judging from the size and staining characteristics of the giant cells observed during this work, it was most probable that the macrophages were the cells of origin.

Mitosis was not observed in any giant cell nucleus although several thousand nuclei of such cells have been scrutinized. This fact, together with the frequent occurrence of mitoses in most other cell types observed, supports the concept that the multinucleated giant cells are formed by fusion of cells rather than by nuclear division without division of cytoplasm.

Epithelium Like Cells

Polygonal cells touching one another and assuming an epithelial appearance were usually found in small patches or sheets surrounded by or interspersed with other cell types. They were first observed in 8 day chambers and both the cell sheets and the individual cells tended to be larger in the older chambers up to the third week; later the fibroblasts were the dominant cell type. In sections as well as in whole mount preparations they sometimes showed a striking resemblance to normal mesothelial cells (Figs. 7–8 and 13, 14). More often they resembled the surface cells seen in regenerating peritoneal wounds (Figs. 24–31).

Sheets of cells closely resembling those of a simple squamous epithelium were also observed in the chambers studied by *Shelton & Rice* (12). The reason why these cells were classified as a special type of fibroblasts is not known.

Illustrations of sectioned filter membranes demonstrating an apparently continuous inner lining of flattened cells similar to those depicted in Figs. 7 and 8 have been published by *Curran* (3).

It was demonstrated in a previous study that the outer surface of intraperitoneal diffusion chambers were covered by a fibrous tissue capsule outermost lined by a complete layer of mesothelium (6). Cells growing within the chambers are nourished by peritoneal fluid seeping through this tissue capsule and through the small pore 150 μ thick filter membrane. Each of these barriers must to a considerable degree impede the exchange of fluid, nutrients and metabolites necessary to sustain the life of the cells within the chambers. It is hardly permissible to claim that any of the cells observed in the present study or in the chambers studied by *Shelton & Rice* are identical with normal meso-

thelial cells The important conclusion is, however that peritoneal fluid cells cultured under these unfavourable conditions may transform to cells of distinct epithelial appearance This fact seems to support the concept that they may, under more physiological circumstances differentiate to mature mesothelial cells

The ultrastructure of peritoneal fluid cells cultured in diffusion chambers will be the subject of a following report (7)

SUMMARY

Autologous peritoneal fluid cells were cultured in intraperitoneal diffusion chambers in rats Histological sections and whole mount preparations of the filter membranes upon which the cells were growing were studied after implantation periods ranging from 2 days to 6 weeks

In addition to the cell types originally present in the inoculum and to some cells which could not be classified with certainty three fairly well defined cell types were observed Fibroblasts, multinucleated giant cells and epithelium like cells The latter resembled normal mesothelial cells or the cells seen on the surface of regenerating peritoneal wounds

The observations are considered to support the concept that peritoneal fluid cells may under more favourable conditions, differentiate to mesothelial cells

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GROWTH OF AUTOLOGOUS PERITONEAL FLUID CELLS IN INTRAPERITONEAL DIFFUSION CHAMBERS IN RATS

2 *An Electron Microscopical Study*

By

GUNNAR ESKELAND and ASMUND KJÆRHEIM

Received 23 v 66

In a previous study (4) autologous peritoneal fluid cells were cultured in intraperitoneal diffusion chambers and examined by light microscopy after varying periods of implantation A variety of cell types were observed, among these were large flat cells of an epithelial appearance and sometimes closely resembling regenerating mesothelial cells

This report describes electron microscopical observations on peritoneal fluid cells cultured under the same conditions We have especially tried to distinguish between macrophages and fibroblasts on the one hand and cells with mesothelial characteristics on the other For this distinction we have used the criteria established in a previous report (3)

MATERIALS AND METHODS

A detailed description of the materials and methods used in the present study has been published previously (4), and only the main points are summarized here

Peritoneal fluid was collected from adult male rats and instilled in diffusion chambers which were implanted intraperitoneally for 2 days to 6 weeks The chambers were made from acrylic rings 2 mm thick and with an inner diameter of 24 mm to which were glued Millipore HA filter membranes[1], pore size 0 45 μ Each chamber had two compartments separated from each other by the central filter membrane The cell compartment contained a thin plastic disc, 4 mm in diameter and was charged with 50 150 cmm autologous peritoneal fluid containing on the average 7 21 million cells The control compartment served to check that the chamber was cell tight

When the chambers were recovered the plastic disc and a piece of the central filter were removed rinsed in 5 per cent glucose and fixed in 1 per cent osmium tetroxide in 0 1 M phosphate buffer at pH 7.3 for 1–2 hours Alternatively fixation took place in 2 5 per cent glutaraldehyde in 0 1 M phosphate buffer at pH 7 3 for 2 hours followed by osmium tetroxide for 1 hour The specimens were kept at 4° C during fixation They were dehydrated in graded acetones and embedded in Vestopal W in such a way that sections could be cut in a direction vertical to the

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This investigation was supported by grants from [illegible] Alexander Malthes Legat and from *The Norwegian Research Council for S[illegible] and the Humanities*

[1] Millipore Filter Corporation Bedford Mass

surface of the plastic disc or filter membrane Thin sections were cut on a Huxley microtome and stained with lead citrate some sections were also stained with 1 per cent uranyl acetate in 30 per cent methanol They were examined at 80 kv in a Siemens Elmiskop I fitted with a double condenser and a 50 μ objective aperture

RESULTS

Cells with the appearance of peritonal macrophages were observed in some chambers throughout the observation period In the early stages they were found apparently unchanged in a single layer on the filter membrane (Fig 1) Later cells of this type were generally larger and had more numerous and longer cytoplasmic processes, sometimes intertwining (Figs 2 and 3) They possessed a higher number of mito chondria and a more prominent endoplasmic reticulum, and many nuclei revealed one or two distinct nucleoli Cytoplasmic vacuoles were common, either empty or filled with phagocytosed material

The cells observed on the plastic discs placed in the chambers were flat and had long slender cytoplasmic processes on the surface (Fig 4) In other respects they resembled macrophages They were usually growing in a single layer and chiefly near the edge of the disc, leaving a large part of the disc surface uncovered

Judging from the amount of cell growth the filter membrane pre sented a far better substratum for the cells than the plastic disc

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All illustrations are electron micrographs of autologous peritoneal fluid cells cultured in intraperitoneal diffusion chambers

Figs 1 3

Fig 1 Peritoneal macrophages in a single layer on the filter membrane of a cham ber 8 days after the implantation N = nucleus × 9 000

Figs 2 3 Round cells of macrophage type were still found in some chambers at 3 weeks These cells had numerous cytoplasmic processes an increased number of mitochondria (MI) and a more prominent endoplasmic reticulum (ER) compared with the normal macrophages × 9 000

Figs 4 7 (see page 504)

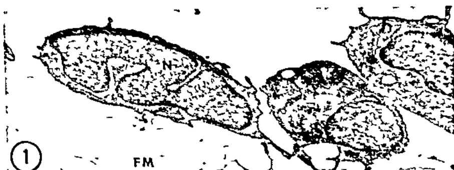
Fig 4 Cell growing on the plastic disc (PL) in a chamber at 3 weeks × 12 000

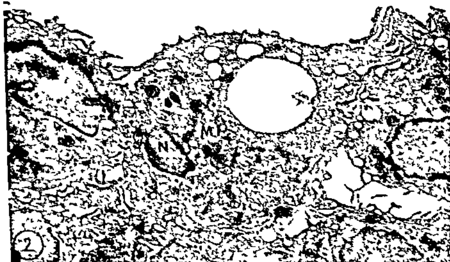
Figs 5–6 A single layer of flat cells on the filter membrane (FM) at 3 weeks Numerous nuclei without intervening cell junctions were seen in these cells which were considered to be multinucleated giant cells Phagocytosed ma terial (P) was found in membrane bounded vacuoles some vacuoles were empty (V) Fig 5 × 8 000 Fig 6 × 10 000

Fig 7 Fibroblasts ? in a chamber at 2 weeks The superficially located cells pos sessed irregular cytoplasmic processes on the surface a prominent rough surfaced endoplasmic reticulum (ER) and fine filaments in the cytoplasm (arrows) × 15 000

Figs 8 10 (see page 505)

Fibroblasts in several layers at 2 and 3 weeks Glycogen granules (G) were present in many cells Collagen fibrils (COL) were discernible at 2 weeks (Figs 8 and 9) and were often abundant at 3 weeks (Fig 10) Fig 8 × 16 000 Fig 9 × 24 000 Fig 10 × 12 000

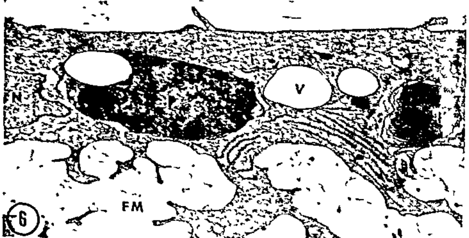

N
FM
1


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3



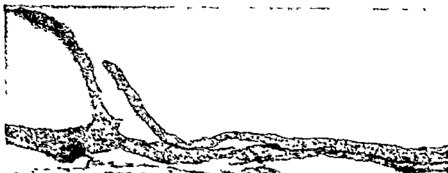






(For text see page 502)


COL




COL


40
COL

On the filter membranes flat cells were sometimes growing in a single layer revealing several nuclei but no discernible partitions between individual cells (Figs 5 and 6) These cells were probably identical with the multinucleated giant cells observed in the light microscopical study (4) They contained large membrane bounded vacuoles usually filled with osmiophilic material probably representing phagocytosed cell debris

The giant cells as well as other types of cells in direct contact with the filter membrane sent long cytoplasmic processes into the pores of the filter often for a distance of 20 30 μ (Figs 5 6 12) These processes were usually devoid of organelles with the exception of profiles of the endoplasmic reticulum which might extend into the projections for a short distance (Fig 6)

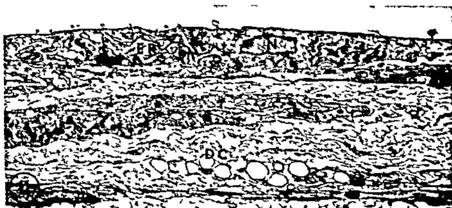
In other areas a few layers of elongated cells with an abundant rough surfaced endoplasmic reticulum were seen (Fig 7) Bundles of fine cytoplasmic filaments occupied the peripheral parts of the cytoplasm in many of these cells which were considered to be fibroblasts In most chambers they grew in several layers parallel to the surface of the filter membrane (Figs 8 13) They were extremely long and flat and were usually separated by a varying amount of intercellular substance Collagen fibrils were demonstrable in the intercellular space after two or more weeks of implantation (Figs 8 10) Fragments of membranes probably representing remnants of dead cells were occasionally seen between the viable cells (Fig 11) In some places the plasma membranes of adjacent cells were running in close proximity for considerable distances (Fig 13) Tight junctions or desmosomes were never observed between cells of this appearance

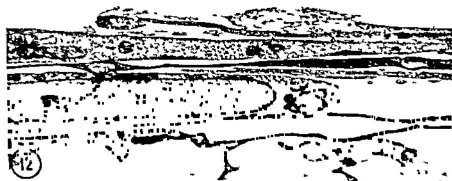
The cells forming the inner lining towards the chamber lumen usually had a smooth surface (Figs 8 and 9) or they displayed small blunt projections from the luminal surface (Figs 10 and 11) The rough surfaced endoplasmic reticulum was well developed (Figs 10 11 13) and some cells also possessed a considerable number of free ribosomes (Fig 13) The mitochondria varied in size from one cell to another as seen in Fig 13 The cell between the arrows had large mitochondria while those of the cell in contact with the filter membrane were small and more dense Empty vacuoles were present in some cells (Fig 13) and glycogen granules in others (Fig 9) Figs 14 16 are electron micrographs illustrating at a higher magnification some of the structures mentioned Collagen fibrils with their characteristic cross band

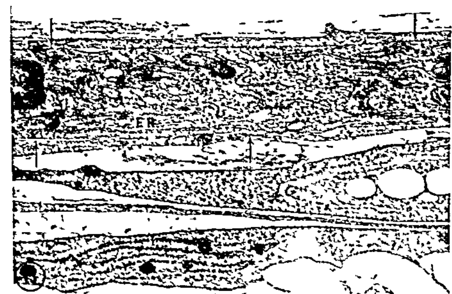
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Figs 11 13

Fibroblasts in chambers at 2 weeks Many cells had extremely long and thin cytoplasmic extensions The endoplasmic reticulum (ER) was well developed and of the rough surfaced type The intercellular space was often very narrow in places only 150–200 Å (arrows) Remains of dead cells (DC) were occasionally seen
Fig 11 × 6 000 Fig 12 × 9 000 Fig 13 × 18 000


ER
N
DC


12


ER


COL
14


C
C
15


COL
16

ing (Figs 14 and 16), bundles of cytoplasmic filaments (Figs 14 and 15), and glycogen granules, which sometimes occupied a greater part of the cytoplasm (Fig 15) In many chambers densely staining fine fibrils were observed in the extracellular space (Fig 16) Although a characteristic periodicity was not demonstrated in these fibrils, they probably represented fibrin

As previously stated, most cells in the layer bordering on the chamber lumen had a smooth surface or small, blunt processes projecting into the lumen Other cells displayed numerous long cytoplasmic processes on the surface (Fig 17) In some instances these processes were extremely long some of them measuring more than 3 μ, and of a fairly uniform diameter of 800–1000 Å (Figs 18 and 19) Processes of this appearance were morphologically indistinguishable from the microvilli of peritoneal mesothelium (3) At high magnification fine filamentous structures were sometimes demonstrable in these microvilli (Fig 19)

Special attention was devoted to the zones of contact between the cells in the luminal layer Cells of macrophage appearance usually abutted end to end without any intimate contact between apposing plasma membranes (Fig 20), or with intertwining of the cytoplasmic processes as illustrated in Fig 2 In most chambers the cells in the lining layer more or less overlapped (Figs 21–23) Usually, there were no specialized sites of contact between such cells, but sometimes an obliteration of the intercellular space was observed near the luminal surface (Figs 22 and 23)

Figs 14–16

Fig 14 Bundles of filaments (arrows) in the cytoplasm of a cell in the luminal layer of a chamber at 3 weeks Numerous collagen fibrils (COL) in the intercellular space × 45 000

Fig 15 Large deposits of glycogen (G) in a deeply situated cell at 2 weeks Fine filaments were present in the peripheral parts of the cytoplasm (arrows) × 24 000

Fig 16 Collagen (COL) and fine fibrils probably fibrin (arrows) in the intercellular space at 3 weeks × 60 000

Figs 17–19 (see page 510)

Cytoplasmic processes in varying numbers were frequently seen projecting from the surface of cells in the luminal layer Their shape and dimensions varied (Fig 17) In some instances they were very long up to 3 μ slender and uniformly wide 800 1000 Å (Fig 18) These features made them appear identical to the microvilli of normal mesothelial cells (3) Fine filaments were sometimes discernible within these microvilli (Fig 19) Fig 17 × 15 000 Fig 18 × 12 000 Fig 19 × 60 000

Figs 20 23 (see page 511)

Zones of contact between neighbouring cells in the luminal layer of chambers at 2 and 3 weeks The cells met end to end or overlapped more or less but specialized junctional complexes were usually not seen In Figs 2 and 23 the plasma membranes of adjacent cells seem to fuse (arrows) this may be due to tangential sectioning N — nucleus L — lipid ER — [illegible] plasmic reticulum
Fig 20 × 15 000 Fig 21 × 60 000 [illegible] 23 × 24 000





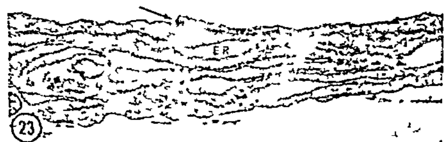


(For text see page 503)









r text x page 509)

In other instances the plasma membranes of contiguous cells were interdigitating, and a tight junction was observed near the free surface (Fig 24) or a desmosome was present at a deeper level (Figs 25 and 26) Cell contacts like these were regularly observed in normal and regenerating mesothelium (3)

DISCUSSION

Rowsell et al (11) found that most of the peritoneal fluid cells in intraperitoneal diffusion chambers died within a few days whether a fibrogenic dust was present or not, and the "corpses" were observed months later In the present study, a certain number of cells died during the first few days after the implantation Later, dead cells were rarely seen, and cell debris appeared to be rapidly phagocytosed The discrepancy between the observations made in the two investigations may possibly be explained by the difference in experimental conditions The filter membranes used by *Rowsell et al* were of a very small pore size (0 1 μ) and filtering area (diameter 12 mm) These properties may have resulted in a filtering capacity insufficient to meet the metabolic demands of the majority of cells Only fibroblasts were observed to grow Secondly, the cells were pooled samples implanted in homologous hosts, and immunologic incompatibility may therefore have affected the cells adversely

The present investigation has confirmed the variability in cell types observed in the chambers by light microscopy (4) After the first week of implantation, 4 fairly well-defined cell types were observed Macrophages, fibroblasts, multinucleated giant cells, and epithelium like cells Some cells were also seen which could not readily be classified

The macrophages were sometimes indistinguishable from normal peritonal macrophages, but they usually exhibited criteria of increased activity Numerous cytoplasmic processes on the surface, an increased number of mitochondria a well developed granular endoplasmic reticulum, and frequently distinct nucleoli

Fibroblasts The elongated cells found in most chambers and illustrated in Figs 8–13 were identified as fibroblasts because of their shape, their long cytoplasmic extensions and prominent rough-surfaced endoplasmic reticulum Collagen fibrils were as a rule demonstrable in the vicinity of these cells Fine cytoplasmic filaments similar to those depicted in Figs 14 and 15 have been observed in fibroblasts under normal and pathological conditions (7, 9), but are not confined to fibroblasts

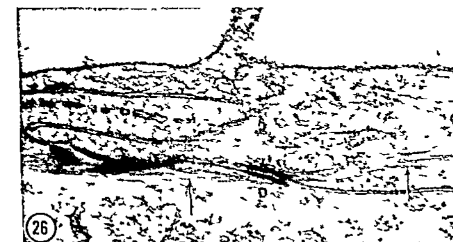
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Figs 24 26

Tight junctions (TJ) and desmosomes (D) were sometimes demonstrable between the cells in the luminal layer Fine filaments (arrows) extended from the desmosome plates into the cytoplasm Fig 24 × 24 000 Figs 25 26 × 60 000


TJ
24


D
25


D
26

At times these cells were stacked upon each other so that their plasma membranes ran in close proximity for a considerable distance (Fig 13) *Such a close relationship is usually not present between fibroblasts in vivo*, but similar conditions have been demonstrated to exist among fibroblasts cultured *in vitro* (6)

Another feature distinguishing these cells from ordinary fibroblasts was the extensive glycogen deposits in the cytoplasm of many cells (Fig 15) Deposits of exactly the same appearance have been observed *in fibroblasts growing in subcutaneous diffusion chambers inoculated* with buffy coat cells (10) The authors considered that the glycogen accumulation might result from an altered metabolism but these cells *might also represent a different cell type In the present study the* largest deposits of glycogen were observed in deeply situated cells and in chambers with abundant growth This may suggest that the glycogen storage was due to an altered cell metabolism

Multinucleated giant cells The large flat cells containing many nuclei illustrated in Figs 5 and 6 were considered to represent sections of multinucleated giant cells Our observations yielded no evidence to decide whether these cells form by fusion of individual cells or by division of nuclei without division of cytoplasm

Phagocytosed material was present within cytoplasmic vacuoles in most of these cells frequently in large masses indicating an extra ordinary phagocytic activity (Fig 6) The presence of substantial amounts of cell debris in some chambers may have been the stimulus triggering giant cell formation

Epithelium Like Cells

Light *microscopical studies have revealed sheets of cells with an* epithelial appearance (4 13) The cells constituting the inner lining of the chambers in the present study sometimes formed a continuous layer with *no intercellular substance between neighbouring cells* Microvilli identical in appearance to those present on the surface of mesothelial cells were observed on the luminal surface of some of these cells These microvilli were morphologically distinct from the irregular cytoplasmic processes of macrophages They were not observed on cells in the deeper layers and their formation is probably related to the presence of free fluid in the chamber The reason why only some cells formed microvilli of this characteristic appearance while the majority of cells did not, *is not clear However* the chambers were partly *filled* by fluid and partly by a gelatinous clot and it seems possible that cells cover d by clot do not form microvilli whereas cells whose surface is bathe l by fluid do *Microvilli are traditionally considered to be* asso ciated with fluid absorption

The t interesting finding from our point of view was the pre *sence of tight junctions* and desmosomes between contiguous cells in

the luminal layer (Figs 24–26) Specialized contact zones of these types normally occur in most epithelia (5) Tight junctions are regularly found in peritoneal mesothelium (3) Desmosomes on the other hand are only occasionally present between normal mesothelial cells more often in regenerating mesothelium (3)

Close association regions sometimes reminiscent of terminal bars were observed between cells claimed to be fibroblasts cultured *in vitro* (2) However the source of fibroblasts was 7 day old granulation tissue explanted and cultured on plasma clots for 24–28 hours before examination Obviously the granulation tissue must have been teeming with sprouting capillaries and there is no hint in the published report to exclude the possibility that the cell junctions studied were not contacts between endothelial cells Close association zones are normal structures between proliferating endothelial cells which in other respects may resemble fibroblasts (1 14)

Apart from the mentioned report close association zones have not to our knowledge been reported to occur between fibroblasts *in vivo* or *in vitro* *Movat* (8) has never observed junctions between fibroblasts

In a previous study (3) the following criteria were found to distinguish normal mesothelial cells from fibroblasts and macrophages 1 long and uniformly thick microvilli 2 tight junctions or desmosomes between contiguous cells 3 numerous pinocytotic vesicles and 4 a basement membrane Of these criteria the first and the second have been demonstrated in the present study Pinocytotic vesicles were not abundant in any of the cells as they are in normal mesothelium and endothelium Such vesicles are often sparse in regenerating mesothelium (3) and endothelium (12), and their number is clearly related to the functional state of the cells A basement membrane has not been convincingly demonstrable in the present study The reason for this is not known but it is conceivable that the environment within the chambers did not offer the proper conditions for this structure to form

The presence of characteristic microvilli on the cell surface and of tight junctions and desmosomes between contiguous cells in our opinion proves that these cells are not fibroblasts These features reveal that cells present in normal peritoneal fluid have the potentiality of transforming to cells which display important if not all characteristics of mesothelium The observations strongly suggest that cells from the peritoneal fluid may under more favourable conditions than those existing in diffusion chambers differentiate to mesothelial cells

SUMMARY

Autologous peritoneal fluid cells were cultured in intraperitoneal diffusion chambers and studied in the electron microscope after varying periods of implantation for up to 6 weeks

Fibroblasts producing collagen and multinucleated giant cells dis

playing a high phagocytic activity were commonly observed in the chambers In the inner layer of cells lining the chamber lumen, cells were observed possessing microvilli which were identical in appearance to the microvilli normally present on peritoneal mesothelial cells Tight junctions and desmosomes were occasionally observed between contiguous cells in this layer

These observations support the concept that cells normally present in the peritoneal fluid have potentialities of differentiation which may under the proper conditions result in the formation of mesothelial cells

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GENERALIZED SHWARTZMAN REACTION

Histopathological Findings in Six Fatal Cases with Widespread Lesions

By

FREDRIK SKJORTEN

Received 1 viii 66

The generalized Shwartzman reaction (GSR) (*Apitz* 1934, *Thomas* 1959) was long considered to be predominantly an experimental phenomenon even though *Apitz* (1934) pointed out the similarity of this phenomenon to human bilateral renal cortical necrosis (*Juhel Renoy* 1886) Lately, many reports have stressed the relationship between the experimental GSR and *clinical conditions characterized by disseminated* intravascular coagulation (*McKay* 1965) Bilateral renal cortical necrosis (*McKay et al* 1953), Waterhouse Friderichsens syndrome (*Margaretten & McAdams* 1958), purpura fulminans (*Hjort et al* 1964) acute haemorrhagic leucoencephalopathy (*Vasland & Barrows* 1962), haemorrhagic enterocolitis (*McKay & Whale* 1955)

The experimental GSR is defined by the events which precipitate it and by the resulting tissue damage The original GSR was produced by injections of bacterial endotoxin but identical changes can be produced by other agents capable of causing intravascular coagulation (*Lee* 1962) A necessary condition for the development of GSR is intravascular formation of fibrin (*Good & Thomas* 1953) leading to occlusion of small vessels in many organs In the kidneys this is particularly severe and causes bilateral renal cortical necrosis which is "the hall mark of the generalized Shwartzman reaction (*Thomas* 1959)

In this paper we present six cases of fatal GSR showing disseminated intravascular coagulation with fibrin formation in small vessels in kidneys as well as in other organs Each case has one or more target organs with histopathological changes identical to those of the kidneys in experimental GSR thrombosis haemorrhage and necrosis The histopathological changes in each organ will be reported and structural details which may be of importance for the understanding of the pathogenesis of GSR will be discussed

MATERIAL AND METHODS

In the six year period July 1 1959 to June 30 [illegible] 5 eleven cases of human GSR were diagn[illegible]ed in 10 700 autopsies perf[illegible] the Department [illegible]f Pathology Ullevaal H[illegible]spital Six cases will be d[illegible] Four cases [illegible] bilateral renal

cortical necrosis and one case of Waterhouse Friderichsens syndrome were excluded from this report because the material available did not permit a detailed analysis of the histopathological changes of GSR in man Case 1 5 and 6 have been included in a previous case report (*Skjorten* 1964 a)

Autopsies were performed 12 to 36 hours post mortem Tissue blocks were fixed in 4 per cent formaldehyde and embedded in paraffin Sections were cut at 5 μ and stained with H + E PTAH and acid picro Mallory (*Lendrum* 1949) or MSB (*Lendrum et al* 1962)

CASE REPORTS

Case 1 L H autopsy No 1639/60

This 55 year old woman consulted a physician some months before death with a complaint of stiff fingers and was given phenylbutazone 300 mg daily for three months Ten days before death she suddenly developed facial oedema headache and anorexia and was admitted to the medical department with pulmonary oedema BUN was 191 mg/100 ml There was oliguria and proteinuria Subsequently she developed fever convulsions and coma BUN rose to 385/100 ml and she expired seven days after admission

Autopsy There was bilateral pleural effusion The heart weighed 410 g and the kidneys 330 g The cut surface of the kidneys showed multiple yellow patches in the cortex surrounded by a narrow haemorrhagic zone Microscopically there was wide spread necrosis of the renal cortex but the medulla was intact The necrotic areas showed widespread fibrin thrombi in glomerular capillaries afferent arterioles and a few interlobular arteries There was marked interstitial leucocytes reaction The adrenals were grossly normal Microscopic examination revealed fibrin thrombi in scattered capsular arterioles (Fig 1) and in sinusoids in the outer zona fasciculata In the zona reticularis there were areas of necrosis and haemorrhage The adrenal veins were patent and contained no thrombi No thrombi were found in sections from lungs liver and heart

Comments The autopsy findings in this patient were those of a typical bilateral renal cortical necrosis, similar to the lesion considered diagnostic of experimental GSR In addition the patient had adrenal changes similar to the findings in Waterhouse Friderichsens syndrome, but less severe

Case 2 B L autopsy No 373/63

A 45 year old man suddenly one night developed joint pains nausea vomiting and diarrhoe In the morning he had chills and stayed in bed At 4 P M he felt sick and was seen by a physician The temperature was 38 2° C but physical examination was negative and there was no rash At 9 P M the patient was desoriented and another physician came to see him Numerous petecchiae in the skin were found and the patient was immediately admitted to Ullevaal Hospital He was dead on arrival A blood culture taken shortly after death showed growth of meningococci

Autopsy There were numerous skin haemorrhages from point sized to about 2 cm i diameter Sections showed stasis in venules and arterioles some of which were completely occluded by fibrin and platelet thrombi while others showed smaller fibrin deposits on the vascular wall surrounded by polymorphonuclear granulocytes The brain showed many petecchiae No sections from the brain were available for microscopic examination A capillary in the neurohypophysis contained a recent platelet aggregate (Fig 2) The heart weighed 430 g Sections showed multiple recent platelet aggregates in small myocardial arterioles Sections from the liver showed large recent platelet aggregates in branches from the portal vein and hepatic artery The kidneys weighed 430 g The cut section showed many petecchiae in the renal parenchyma Microscopic examination revealed fibrin thrombi in one or more capillaries of all glomeruli seen in sections from both kidneys (Fig 3) The afferent arterioles were dilated and one contained a recent platelet aggregate No other thrombi were seen in this location Interlobular arteries were not thrombosed The medulla showed stasis and small haemorrhages with platelet aggregates and small

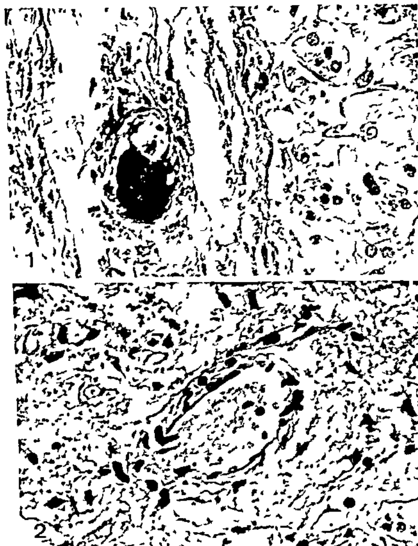


Fig 1 Case 1 Adrenal capsule Thrombosed arteriole PTAH 480 ×
Fig 2 Case 2 Neurohypophysis Platelet aggregate in small vessel MSB 480 ×

fibrin deposits in a few veins One medullary vein contained a hyaline microthrombus (*Skjörten* 1964 b) Both adrenals showed gross haemorrhages Microscopic examination showed widespread haemorrhages in the medulla and the inner half of the cortex The sinusoids surrounding the haemorrhagic areas showed some fibrin deposition but were not completely thrombosed However massive haemorrhages obscured structural details

Comments Rapidly developing meningococcal septicaemia led to death in less than 24 hours Autopsy findings were characteristic of the Waterhouse Friderichsens syndrome In addition there was extensive

fibrin deposition in glomerular capillaries, similar to the findings in the GSR but cortical necrosis was lacking The afferent arterioles and interlobular arteries were patent Small vessels in the renal medulla the skin the heart and the pituitary were occluded by fibrin or recent platelet thrombi

Case 3 E P autopsy No 705/65

This 62 year old woman had been hospitalized 11 years previously because of bilateral otitis She had then developed acute facial oedema after a penicillin injection On the day of admission she became acutely ill and was admitted to the medical department with a temperature of 40 3° C. A chest x ray showed possible pneumonia and a blood culture grew pneumococci On the day of admission she was given two intramuscular injections of 1 mill I U crystalline penicillin During the following night she developed shock and bluish discolourations ecchymoses and petecchiae in face extremities and anterior chest wall The distal 2/3 of all fingers became discoloured A dermatologist diagnosed allergic vasculitis—purpura ful

level of fibrinogen) cephalin time 78 sec (normal 55–65 sec) The platelet count remained low until death

Autopsy There was gangrene of the tip of the nose of all finger tips of a fist sized area on the left breast and of limited areas on the back side of both legs There were also symmetrical skin haemorrhages over both shoulders both breasts abdomen and thighs Sections from the skin showed necrosis of epidermis and dermis but intact subcutis The surrounding skin showed widespread thrombosis of capillaries venules and occasional small arteries in the dermis and subcutis Some vessels were occluded by hyaline fibrin thrombi others by fibrin platelet thrombi containing many polymorphonuclear granulocytes A thrombus in a finger artery showed an irregular outline with vacuoles around leucocytes indicating fibrinolysis (*Jørgensen et al* 1966) The brain was grossly normal Sections showed occasional hyaline microthrombi The right lung showed lobar pneumonia grossly and microscopically with abundant fibrinous exudate in the alveoli Some alveolar capillaries were occluded by fibrin thrombi There was a chronic pancreatitis and pancreatic vessels contained many hyaline microthrombi The kidneys showed typical bilateral cortical necrosis grossly and microscopically with fibrin thrombi in cortical arterioles and glomerular capillaries and advanced necrosis of cortical tissue There were signs of organization in some glomerular tufts The adrenals were grossly normal but sections showed small areas of recent necrosis and haemorrhage in the cortex Surrounding sinusoids contained small fibrin thrombi and recent platelet aggregates (Fig 4) Many medullary veins contained large quite recent platelet thrombi almost devoid of fibrin (Fig 5) The spleen weighed 27 g and appeared atrophic Sections showed almost complete replacement of cellular constituents in the germinal centres by extravasal fibrin The red pulp also showed focal accumulations of fibrin threads Sections from a lymph node showed loose fibrin thrombi in lymph sinuses and scattered fibrin threads in the pulp

Comments A 62 year old woman who previously had developed facial oedema after penicillin injections was given two injections of penicillin because of pneumococcal septicaemia In the course of a few hours she developed shock became anuric and developed extensive symmetrical skin haemorrhages and necroses with thrombosis of small vessels The kidneys showed bilateral cortical necrosis and the adrenals showed small haemorrhages *and vascular occlusions by platelet and*

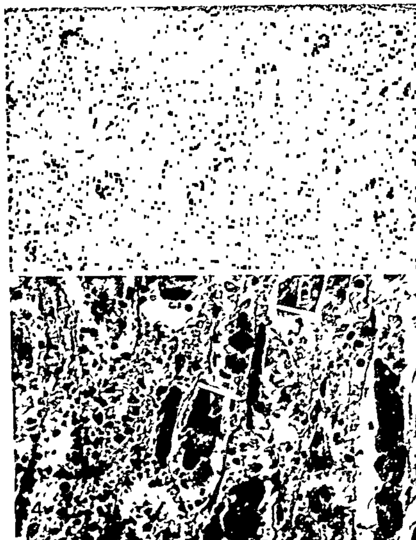


Fig 3 Case 2 Kidney Thrombosis in all visible glomeruli No cortical necrosis PTAH 30 ×

Fig 4 Case 3 Adrenal cortex In sinusoids homogenous fibrin thrombi and adjacent to these, aggregated platelets (arrow) Haemorrhage Necrosis of cortical epithelium PTAH, 480 ×

fibrin thrombi These findings indicate that the patient had been the victim of a GSR, with kidneys, adrenals and skin as target organs

Case 4 I.L autopsy No 89 65

This previously healthy, 52 year old woman suddenly developed headache and dizziness four weeks before death There was some improvement after two days in bed One week later slight temperature elevation was noticed, increasing gradually

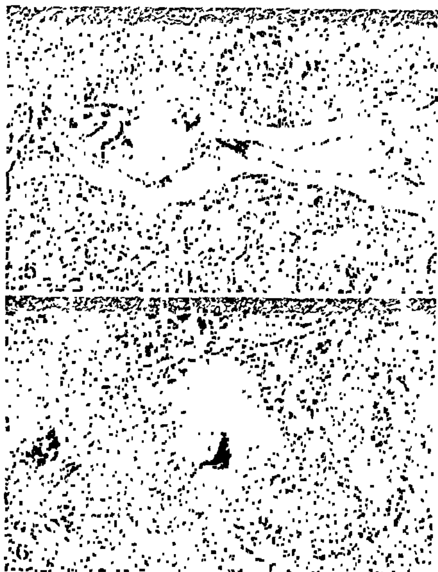


Fig 5 Case 3 Adrenal vein Platelet thrombus with small amounts of fibrin in some areas MSB, 75 ×

Fig 6 Case 4 Brain Centre thrombosed small vessel in white matter surrounded by small anaemic infarcts without cellular reaction PTAH 75 ×

ous breathing stopped and she was subsequently ventilated with a respirator Three days before death she developed severe watery diarrhoea, but stool cultures showed

growth of normal intestinal flora She had been given no antibiotics in the hospital A blood culture was sterile She died two weeks after admission

Laboratory tests Platelet count four days before death 131 000 per c mm, one day before death 19 000 per c mm

Autopsy The brain weighed 1310 g The meninges were grossly normal Cut sections of the fixed brain showed multiple petecchiae in the white matter around the capsula interna, in the corpus callosum, mesencephalon and medulla oblongata down to the upper cervical spinal cord There were no gross lesions in the grey matter Microscopically there were multiple small infarcts in the white matter, some of [illegible]

contained hyaline microthrombi The lungs and liver showed acute congestion The heart was normal Microscopically there were no fibrin deposits in renal vessels Several thoracolumbar vertebrae showed large, white areas grossly Microscopically these were areas of bone necrosis with intense inflammatory reaction predominantly polymorphonuclear In some areas beginning callus formation was seen A prevertebral vein was occluded by a fibrin thrombus No thrombi were seen in the areas of bone necrosis

The colon had greatly thickened mucosa with small ulcerations particularly in the proximal part, similar to ulcerative colitis Microscopically there was great thickening of the intestinal mucosa with multiple areas of haemorrhagic infarction surrounded by inflammation There were scattered small superficial ulcerations A large number of vessels in the mucosa and submucosa were occluded by platelet thrombi Other vessels in the same area contained hyaline microthrombi The adrenals were grossly not remarkable but no sections were available for microscopic examination

Comments A woman in good health suddenly developed dizziness, followed by fever and neurological symptoms Three days before death she developed watery diarrhoe, but stool cultures grew only ordinary intestinal bacteria, and a blood culture was sterile The autopsy showed haemorrhagic leucoencephalopathy, haemorrhagic colitis and necrosis of thoracolumbar vertebrae The cause of the lesions in the brain and intestine was widespread thrombosis of small vessels, similar in appearance to the vascular occlusions in GSR

Case 5 L R autopsy No 1422 61

This 22 year old woman had no family history of epilepsia and had never had convulsions She was admitted to the surgical department one week before death with fever and flank pain She had not felt quite well for the last two weeks and had had urinary frequency and dysuria for 24 hours The temperature was 39 1° C. The abdomen was soft but there was deep tenderness in the right renal region Urine analysis showed proteinuria and many WBC In the hospital she was febrile in spite of antibiotic treatment An IVP three days after admission was essentially normal The next day she unexpectedly developed generalized convulsions and involuntary urination On neurological examination four hours later she was awake but desoriented There were no focal symptoms Subsequently she was somnolent restless and had generalized myoclonus There were no paresis but the plantar reflexes were extensor bilaterally She died suddenly three days after the onset of convulsions seven days after admission

Autopsy The brain weighed 1510 g There was some blood over both frontal lobes Microscopic examination showed small haemorrhages in the meninges and platelet fibrin thrombi in a few small meningeal vessels The white matter showed small perivascular haemorrhages around congested vessels Scattered vessels showed perivascular accumulation of yellow pigment, other vessels were surrounded by a rim of lymphocytes Many intracerebral vessels contained hyaline microthrombi The heart was normal The liver showed moderate degree of nonspecific cellular damage

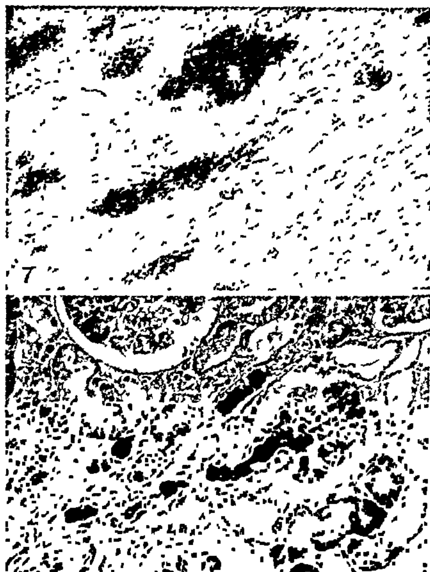


Fig 7 Case 4 Brain Numerous small haemorrhages in white matter H + F 75 ×
Fig 8 Hyaline *microthrombi in kidney of 50 year old man who died from* pneumonia Some hyaline microthrombi lie in arterioles as separate balls Others in afferent arterioles seem to fuse and are continuous with ordinary thrombus in glomerular capillaries PTAH 190 ×

The adrenals were not remarkable grossly Sections were not available for microscopic examination The right kidney showed an egg sized infarct The remaining kidney tissue on both sides showed confluent haemorrhagic areas in the cortex Microscopically some glomerular capillaries were occluded by hyaline *microthrombi* surrounded by a less compact fibrin mesh The majority however were

weeks

Comments This patient was admitted to the surgical department with urinary symptoms Four days later she suddenly developed convulsions She died seven days after admission The autopsy revealed a renal infarct and bilateral renal cortical necrosis The brain showed small subarachnoidal haemorrhages and thrombosis of meningeal vessels as well as scattered small perivascular haemorrhages in the white matter The microscopic appearance of the renal infarct was compatible with an age of one or two weeks but all other lesions appeared quite recent The renal cortical necrosis must have developed within the last three days because her urinary output was normal before this The episode of intravascular coagulation which led to renal cortical necrosis probably also caused thrombosis of meningeal vessels and haemorrhages resulting in convulsions

Case 6 L W autopsy No 1891/61

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separation of the placenta with massive haemorrhage during delivery at least 2000 ml She was anuric from the time of delivery and was therefore transferred to the medical department During the next four weeks four haemodialyses were performed The patient remained anuric One week before death she developed staphylococcal septicaemia She expired four weeks after delivery Blood cultures showed growth of staphylococcus aureus

Autopsy The brain weighed 1350 g There were multiple small abscesses in the [illegible]

unspecific toxic damage The adrenals were grossly normal but microscopic examination showed lipid depletion and small haemorrhages in the cortex No thrombi were seen in sections from both adrenals The right kidney was shrunken and showed a severe chronic pyelonephritis Both kidneys showed cortical necrosis with cortical arterioles and glomerular capillaries occluded by fibrin thrombi Microscopically there was loss of cellular details and partial liquefaction of the renal cortex compatible with an age of the lesion of about four weeks The left renal artery and vein were occluded by platelet fibrin thrombi The vessels to the right kidney were patent

Comments This 41 year old woman had shown signs of toxaemia during two previous pregnancies She was now admitted with premature separation of the placenta She was completely anuric until she died from staphylococcal septicemia four weeks later The autopsy showed bilateral renal cortical necrosis similar to the renal lesion in experimental GSR In addition there was thrombosis of the left renal artery and vein This was thought to be secondary to the extensive liquefaction necrosis of this kidney

DISCUSSION

A *Pathological Findings in Various Organs*

The six cases presented above exhibit signs of intravascular coagulation in several organs with localization and intensity varying from case to case The pathological findings are summarized in Table 1 They will be discussed below

TABLE 1
Distribution of Lesions in Various Organs

Case No	1	2	3	4	5	6
Kidneys	TNH	THh	TNH		TNHh	TNH
Adrenals	TNH	TNH	TNH			H
Brain & meninges		H		TNHh	THh	h
Intestine				TNHh		
Skin		TH	TNH			
Skeleton				N		
RES			T			
Pituitary		T				
Liver		T				
Pancreas			h			
Heart		T				

T = thrombosis N = necrosis H = haemorrhage h = hyaline microthromb
Target organs show TNH or TNHh

Kidneys

Cases 1 3 5 and 6 showed the typical picture of bilateral renal cortical necrosis as first described by *Juhel Renoy* (1886) in human subjects and by *Apitz* (1934) in the experimental GSR Occlusion of glomerular capillaries afferent arterioles and interlobular arteries by fibrin thrombi and widespread necrosis of renal cortical tissue while the medulla always was intact Case 2 showed extensive thrombosis of almost all glomerular capillaries but there was no extension of thrombi to afferent or interlobular arterioles and no necrosis of renal tissue The patient had not however been ill long enough for necrosis to be microscopically detectable Similar glomerular thromboses without cortical necrosis as a cause of uraemia have been reported by *Skjorten* (1964b) Case 4 had no renal lesion

Adrenals

Only case 2 had gross changes in the adrenals Sections were available from four cases Case 1 2 and 3 showed fibrin thrombi in small capsular arterioles (Fig 1) and cortical sinusoids (Fig 4) similar to the findings reported by *Bohle & Krecke* (1959) Case 3 also showed quite recent almost fibrin free platelet thrombi in medullary veins (Fig 5)

Case 6 had recent bilateral adrenal haemorrhages which might well

have occurred during the terminal episode of septicaemia No thrombi were found The epithelial cells in the zona fasciculata were atrophic and had no lipids indicating that severe stress had preceded death Adrenal haemorrhages after severe stress unaccompanied by thrombosis have been reported by *Selye & Stone* (1950)

Brain

Case 4 showed widespread thrombosis of vessels in the white matter surrounded by haemorrhage and necrosis (Figs 6 and 7) The vascular lesions were similar to other manifestations of disseminated intravascular coagulation discussed above *Waksman & Adams* (1957) obtained identical lesions in rabbits with allergic encephalomyelitis and subjected to the classical GSR

Masland & Barrows (1962) reported a case of haemorrhagic leucoencephalopathy with simultaneous occurrence of massive fibrin deposition intravascularly in the kidneys and the brain The lesions seen in this case were thought to be the result of a GSR The lesions in our case 4 are similar to these with massive fibrin deposition in many vessels The course of disease however was more protracted in our case

Case 5 had platelet fibrin thrombi in some meningeal vessels surrounded by haemorrhage and a few small perivascular haemorrhages in the white matter *Waksman & Adams* (1957) frequently observed thrombosis in and haemorrhage around meningeal vessels as a part of a GSR but usually in relation to coexistent allergic encephalomyelitis Case 6 had only small meningeal and intracerebral abscesses related to her staphylococcal septicaemia

Intestine

Case 4 had haemorrhagic leucoencephalopathy and a lesion in the bowel which microscopically showed multiple confluent areas of haemorrhagic necrosis of the mucosa Both lesions were caused by widespread occlusion of small vessels by platelet fibrin thrombi McKay has established the relationship between haemorrhagic necrosis of intestinal mucosa and intravascular coagulation in man (*McKay et al* 1953 *McKay & Whale* 1955 *McKay* 1965) and *Hardaway et al* (1956 1961) have shown that intraaortic infusion of incompatible blood or bacterial endotoxin in dogs cause intravascular coagulation with occlusion of small mucosal vessels by thrombi and haemorrhagic necrosis of intestinal mucosa

Even though the gross picture of acute haemorrhagic colitis may be similar to ulcerative colitis as in our case the microscopic picture is totally different from that of fulminating ulcerative colitis which lacks thrombosis of small vessels

Skeletal System

Case 4, in addition to haemorrhagic leucoencephalopathy and haemorrhagic colitis also showed necrosis of several vertebrae No thrombi were seen in the necrotic areas, but a prevertebral vein near by was occluded by a fibrin thrombus The microscopic picture was compatible with, but not diagnostic of an ischaemic bone necrosis of several weeks duration *Nilsson et al* (1961) have reported a case of ischaemic necrosis of several vertebrae caused by intravascular coagulation

Skin

Case 3 who showed renal lesions typical of GSR, had widespread symmetrical skin necroses with thrombosis of small vessels in the dermis and of occasional subcutaneous vessels The skin lesions in this case were similar to the skin lesions in purpura fulminans (*Hjort et al* 1964) Case 2 had a typical Waterhouse-Friderichsens syndrome with scattered skin haemorrhages and fibrin platelet thrombi in small skin vessels, without necrosis

Reticulo Endothelial System

Case 3 had a spleen which was about 1/5 of normal size and showed extensive fibrin deposition in germinal centres and red pulp Lymph nodes in this case showed fibrin thrombi in lymph vessels and sinuses and reticular fibrin deposited in the pulp No necrosis was seen in the reticulo endothelial system (RES)

In summary, the essential histopathological lesion in all cases discussed above is similar to the typical lesion of the experimental GSR massive occlusive thrombi in small vessels, with necrosis and haemorrhage These lesions are localized in *target organs* which may vary *according to the experimental or clinical conditions In the experimental* GSR in rabbits (*Apitz* 1934) the kidneys are target organs In the experimental model of *Waksman & Adams* (1957) the brain and kidneys are target organs In human GSR the kidneys (*McKay et al* 1953), adrenals (*Margaretten & McAdams* 1958), brain (*Masland & Barrows* 1962), intestine (*McKay & Whale* 1955), skin (*Hjort et al* 1964) and possibly skeletal system, or a combination of these locations may be target organs

The vascular lesions in human and experimental GSR are widespread (*McKay et al* 1953, *Thomas* 1959) and are not restricted to the target organs In our cases, thrombosis of small vessels, not accompanied by necrosis, were found in kidneys, meninges skin RES pituitary, liver and heart (Table 1) Some early lesions in these organs were pure platelet thrombi, but older lesions seemed to consist mainly of fibrin thrombi —The transformation of platelet to fibrin thrombi takes place within one to a few days (*Jorgensen* 1964, *Jorgensen et al* 1966) — Hyaline microthrombi were frequently found

B Aetiology and Pathogenesis

The experimental GSR is classically produced in young rabbits by two intravenous injections of gram negative bacterial endotoxin given 24 hours apart (*Apit* 1934) The reaction is prevented by heparin (*Good & Thomas* 1953) Thrombin infusions with or without RES blockade or cortisone treatment may produce a reaction which is histologically identical to the classical GSR (*Lee* 1962) In pregnant rhodents a similar reaction will occur at term when the animals have been given a tochopherol deficient diet rich in oxydized lipids (*McKay & Wong* 1962) Thus it seems that any experimental procedure which causes massive intravascular fibrin formation can under suitable condition give GSR

In human GSR the events which precipitate intravascular coagulation are equally diverse, and may at times be difficult to elucidate In two of the cases presented here (case 4 and 5), the material available for study failed to reveal any clue to the triggering event Case 6 had premature separation of the placenta In older publications (*Duff & More* 1941) premature separation of the placenta was the most common cause of bilateral renal cortical necrosis but improved antenatal care and better hospital facilities has made it a rare cause of human GSR

Case 2 had meningococcaemia The clotting disturbance in this condition is probably triggered by the release of large amounts of endotoxin into the systemic circulation (*McKay* 1965) Therefore meningococcaemia may be the clinical condition which most closely mimics the classical experimental GSR

In case 3 two aetiological factors may have contributed to the development of the GSR The patient was sensitive to penicillin Allergic reactions to penicillin in previously sensitized persons may take place after a latent period of a few hours to two or three days with vascular cutaneous or visceral manifestations (*Siegal* 1960) *Walter & Frank* (1961) demonstrated fibrin emboli in pulmonary vessels of rabbits subjected to anaphylactic shock and *Lee* (1963) triggered GSR by injecting protein antigens into specifically immunized rabbits However the patient also had pneumococcal septicaemia *Blackman & Rake* (1939) reported bilateral renal cortical necrosis in three infants dying from severe pneumococcal infections *Ratnoff & Nebehay* (1962) reported a case of Waterhouse Friderichsens syndrome in a 17 year old woman with pneumococcal septicaemia who had previously been splenectomized Therefore it is possible that either allergy or infection or both may have triggered the GSR in this patient In case 1 the GSR occurred after three months of phenylbutazone therapy This drug is known to give toxicity reactions (*Tungland & Skjorten* 1962) In this patient an allergic reaction to phenylbutazone may have triggered the GSR

Previous publications have given different criteria for th

of human GSR *Bohle & Krecke* (1959) used aetiological criteria and diagnosed as GSR cases of disseminated intravascular coagulation with an infectious aetiology *McKay* (1965) used morphological criteria, but restricted the diagnosis human GSR to case actually showing bilateral renal cortical necrosis, and classified GSR with target in other organs as disseminated intravascular coagulation In our opinion, disseminated intravascular coagulation is a common reaction pattern in human disease, with diverse morphological features, which includes GSR GSR stands out from other types of disseminated intravascular coagulation by the severity of intravascular fibrin formation in the target organs, which leads to necrosis Necrosis is always present in the target organs if the individual has survived long enough for necrosis to manifest itself, grossly or microscopically Necrosis, therefore, is an important morphological criterion in the differentiation between GSR and other types of disseminated intravascular coagulation

A striking feature of GSR is the symmetrical distribution of lesions, particularly when paired organs are targets of the phenomenon In the kidneys and adrenals there is always bilateral affection Equally striking is the symmetrical localization of skin lesions frequently seen in purpura fulminans Our Case 3 also showed symmetrical skin lesions

The tendency to symmetrical distribution of tissue changes in GSR may be a clue to the understanding of the pathogenesis of the phenomenon The conditio sine qua non of GSR is intravascular fibrin formation Where the fibrin thus formed will localize, depends on several local factors

1 *The anatomy and physiology of the microcirculation in an organ or part of an organ* In the renal cortex arteriovenous shunts may operate in shock (*Truela et al* 1949) and lead to reduced glomerular perfusion This may facilitate thrombosis during disseminated intravascular coagulation

2 *Pathological alterations in vessel's walls* *Waksman & Adams* (1957) have investigated this problem They tried to produce acute haemorrhagic leucoencephalopathy by subjecting rabbits to the GSR, but got no cerebral lesions When they, however used rabbits which had previously been immunized with brain tissue, thrombosis and necrosis occurred in relation to preexistent vascular-perivascular lesions typical of allergic encephalomyelitis In addition, the rabbits developed typical glomerular thromboses In this situation the preexistent allergic encephalomyelitis served to localize fibrin in the cerebral vessels

3 *Vasomotor changes* Case 3 had gangrene of all fingers and the tip of the nose caused by fibrin thrombi in small arteries and arterioles The development of the skin lesions was preceded by or accompanied by shock It seems possible that vasomotor changes related to the shock may have slowed down the circulation in fingers and nose and thus served to localize fibrin here *Palmerio et al* (1962) demonstrated

the importance of vasomotor factors for the development of GSR They showed that denervation of the renal pedicle prevented experimental GSR in rabbits

The quantity of fibrin formed is determined by the state of function of the RES When the RES is blocked, for instance by thorotrast, one injection of endotoxin or thrombin is sufficient to produce GSR (*Good & Thomas* 1952, *Lee* 1962) According to *Lee* (1962), the fibrin formed after the first endotoxin injection is taken up by the RES, which consequently is blocked at the time of the second injection, when fibrin is again formed This fibrin is then deposited in small vessels *Prose et al* (1965) have demonstrated the actual uptake of fibrin by RES cells in the liver

Our case 3 had a spleen which was about 20 per cent of normal size The capacity of the RES was therefore probably reduced This may have contributed to the very violent GSR which this patient showed The material available for study did not permit any conclusion as to the state of function of the RES in the other cases reported

When the release of fibrin precipitating agent into the circulation is massive enough, fibrin will always be formed intravascularly *Robbins & Collins* (1961) and *Vassali et al* (1964) injected thrombin into the aorta or renal artery and obtained tissue changes identical to the renal lesions of the experimental GSR

Hyaline microthrombi were first reported by *Welch* (1889) and elaborated by *Apitz* (1942) In two publications (*Skjorten* 1964 a and b) we proposed the theory that hyaline microthrombi might be the primordial body in conditions of intravascular coagulation and when embolized into glomerular capillaries lead to thrombosis and GSR This theory was based on the observation of hyaline microthrombi surrounded by fibrin thrombi in glomeruli of one patient (case 5) Subsequently we have observed similar localization of hyaline microthrombi in another case with quite extensive glomerular thromboses but no cortical necrosis (Fig 8) It must be emphasized, however, that the occlusion of glomerular capillaries by hyaline microthrombi is a rare finding, and not the mechanism leading to glomerular thrombosis in the majority of cases of GSR

C *May Human GSR Be the Result of More than One Episode of Intravascular Coagulation?*

In three of the cases presented in this paper, the case histories and microscopic findings indicate that the GSR may have developed in two or more steps The adrenals of case 3 showed signs that the thrombi had developed in two steps Capsular vessels and sinusoids contained fibrin thrombi similar to those seen in the skin and kidneys The medullary veins however, contained quite recent platelet thrombi with very little fibrin

Case 4 had a stepwise development of neurological symptoms, leading to death in four weeks Microscopically the brain showed infarcts and haemorrhages of different age, some quite recent and some older, but compatible with a history of four weeks duration The cellular infiltrate surrounding the infarcts in the intestinal mucosa had an appearance compatible with three or four days duration These findings indicate that several episodes of intravascular coagulation had taken place

Case 5 had a renal infarcts surrounded by a cellular infiltrate which showed dissolution and fragmentation of leucocytes, therefore assessed to be at least five days old Clinically she had normal urinary output until three days before death, and the cellular infiltrate, surrounding the areas of cortical necrosis showed intact leucocytes in moderate number, compatible with an age of no more than three days for the renal cortical necrosis The thromboses and haemorrhages in the meninges were of the same age

Purpura fulminans (*Hjort et al* 1964) is also characterized by a protracted course, with several exacerbations of intravascular coagulation

Thus, there is indication that human GSR need not develop in a single event, but may have a protracted course, with several episodes of intravascular coagulation This forms the basis for the advocation of anticoagulant therapy in patients with GSR Anticoagulant therapy may prevent further episodes of intravascular coagulation, and thus save the patient if irreparable damage has not already been done by the time therapy is instituted

SUMMARY

Six fatal cases of human generalized Shwartzman reaction (GSR) are reported The reported cases show histopathological findings similar to the findings in the experimental GSR

Human GSR stands out from other types of disseminated intravascular coagulation by the intensity of intravascular fibrin formation which causes vascular occlusions and necrosis in one or more target organs The target organs as well as the extent of tissue damage, may vary from case to case The demonstration of necrosis aids the pathologist in differentiating human GSR from other types of disseminated intravascular coagulation

Cases of human GSR may have a diverse aetiology The reaction may be precipitated by any situation which causes massive intravascular coagulation It is pointed out that the essential lesion in all cases is thrombosis of small vessels The localization of thrombi is determined by vascular factors

The reported cases support the assumption that more than one episode of intravascular coagulation may take place in human GSR

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A SPONTANEOUSLY METASTASIZING 20 METHYLCHOLANTHRENE INDUCED RHABDOMYOSARCOMA AND ITS TRANSFORMATION TO ASCITES FORM IN THE CBA MOUSE

By

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Received 20 vi 66

Many transplantable and transmissible tumours extensively used in experimental oncology are fairly old (*Flexner & Jobling* 1907, *Jensen* 1909, *Brown & Pearce* 1923a, *Loewenthal & Jahn* 1932, *Earle* 1935, *Kidd & Rous* 1940) After a varying number of transplant generations, however, the majority of tumours tend to change their properties, displaying decreased pleomorphia (*Stewart et al* 1959), increased growth rate, and decreased life span of the host, which may complicate observation of metastases (*Ostenfeld* 1941)

With the exception of lymphomas and leukaemias, few transplantable tumours in the mouse metastasize regularly or frequently (*Dunham & Stewart* 1953, *Stewart et al* 1959)

Important investigations on tumour cell dissemination have been performed with Walker carcinosarcoma (*Earle* 1935, *Warren & Gates* 1936, *Sugarbaker* 1952, *Schmahl & Rieseberg* 1958, *Cliffton & Agostino* 1962 *Saldeen* 1962, *Fisher & Fisher* 1965 etc) V2 carcinoma (*Kidd & Rous* 1940, *Zeidman & Buss* 1952, *Cliffton & Grossi* 1956 *Wood* 1958 1964 etc), Flexner-Jobling carcinoma (*Flexner & Jobling* 1907, *Sugarbaker* 1952, *Zeidman et al* 1956 etc), Jensen sarcoma (*Jensen* 1909 *Dryckrey et al* 1939, 1949 etc), Brown-Pearce carcinoma (*Brown & Pearce* 1923 b *Zeidman & Buss* 1952, *Cliffton & Grossi* 1956) Rous sarcoma (*Saldeen* 1962, 1963), and Ehrlich ascites tumour (*Ambrus et al* 1956 *Selecki* 1959) None of these tumours has been used in an isologous system however, and therefore immunological factors may have influenced the results

Klein (1959) emphasized the importance of an isologous tumour host system and the value of recently arisen spontaneous or induced tumours for securing as far as possible genetic identity within the system

This investigation was supported by research grants from the *Swedish Cancer Society*

To improve the means for studying metastases we tried to induce, in solid form, a tumour with a high spontaneous metastatic rate and also to transform it to ascites form The results was the rhabdomyosarcoma to be described

Induction and Selection of Tumour

From one littermate pair of inbred CBA mice, delivered from G Klein Institute of Tumour Biology, Stockholm, in 1960, one pedegree line has been propagated in this laboratory by brother sister mating Inbred mice with common ancestors no more than 4 generations earlier in the pedegreed strain were used for all experiments (cf *Green* 1962)

One series of 11 mice of both sexes were given 1 mg 20-methylcholantrene in 0 1 ml sesam oil subcutaneously in the shoulder region on September 17, 1960 Another series of 13 mice of both sexes was treated in the same way twice on September 17 and November 3 1960 In every animal—except one which died within 108 days—a palpable tumour was observed within 85 to 167 days after the first injection somewhat earlier in animals treated twice When the tumour had grown to a diameter of 1 to 2 cm the animals were killed Small pieces of each tumour were transplanted subcutaneously to the groin of at least 3 mice in successive transfers with 100 per cent takes The entire lungs and pieces of the liver from all animals were searched histologically for metastases

Among those tumours with a high growth rate through the second and third transfer generations the one was chosen which had the largest metastasis rate to the lungs This tumour appeared in a male mouse injected twice with methyl cholantrene and had a diameter of about 1 5 cm on December 15, when the animal was killed Since then it has been transplanted every 10 30 days subcutaneously to the groin in pieces introduced by a trocar or by injection of 0 05–0 1 ml of tumour mince

Properties of the Solid Tumour Form

Growth rate survival time of the host frequency of spontaneous metastases observable in histological sections of lungs [illegible] types of the tumour were investigated du[illegible] and May 1965 The results in 1961 and 1963 [illegible]

Thus in 1963 after 39 transfers there w[illegible] after 3–7 days (mince) 4–7 days (pieces) kills host in 28 49 days Metastases to lungs (100 per cent 23 days 50 per cent 17–22 days) occasionally to regional lymph nodes never to liver Appearance of tumour gross Solid soft white nodular mass Microscopic Tumour cells of varying size some elongated, some rounded often multinucleated contain myofibrils without cross striation branching common and arrangement irregular nuclei irregular in size and shape with prominent nucleoli mitoses frequent stroma delicate vascular marginal infiltration central necrosis (Figs 1 2 3 4)

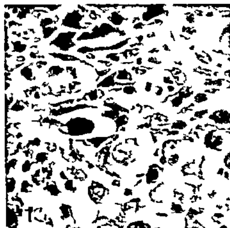
In 1965 after 101 transfers takes and growth rate as above but kills host in 10 34 days average 18 days Metastases to lungs (100 per cent 24 days 30 per cent 17 23 [illegible]

[illegible]

scarce (Fig 5)

Figs 1–7

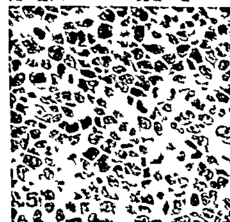
Fig 1 MCGI SS Dec 1960 (v G × 400)
Fig 2 MCGI SS Dec 1960 (v G × 1200)
Fig 3 Spontaneous pulmonary metastases (v G × 120)
Fig 4 Spontaneous liver metastases (Htx eosin × 120)
Fig 5 MCGI SS May 1965 (v G × 400)
Fig 7 MCGI AA 30th transfer generation (Papanicolaou × 400)


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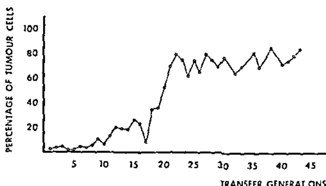


Fig 6

Transformation of MCGI to ascites form

Specificity test on C57BL/10J 0 1 ml mince subcutaneously 1/5 one mouse developed a tumour after 5 months

The tumour was interpreted as a rhabdomyosarcoma (cf *Green* 1962 *Homburger & Fishman* 1953) and denoted MCGI whose solid form—described so far—was denoted SS form (cf *Klein* 1955 a)

Vitality of tumour cell suspension produced according to *Madden & Burk* (1961) with minor modifications (*Boeryd et al* 1965) and critical inoculum dose of MCGI SS were tested by subcutaneous injections 218 injections 0 05 ml in modified Parker 199 (*Salk et al* 1954 SBL Stockholm Sweden) were given to 23 CBA mice aged 3 4 weeks with the following number of cells (statistically anticipated after dilution) 3600 100 per cent takes 400–100 per cent 30 60 per cent 3 20 per cent 2 10 per cent and less than 2 cells 0 per cent Actual viability was 90 per cent by vital dye test Cell viability of a similar suspension has been tested by nucleic acid turnover in correlation to Trypan Blue and Neutral Red tests (*Boeryd et al* 1965) Intravenously injected 50 000 cells 100 per cent takes after 18 days 10 000 cells 80 per cent takes after 23 days

Transformation to Ascites Form

Attempts on 42 animals to convert the tumour to ascites form by inoculation of tumour mince intraperitoneally according to *Klein* (1951 a) or by injecting 34 animals with cell suspension according to *Boeryd et al* (1965) were unsuccessful Tumour mince was then transplanted to one testis in each of 27 mice most of which survived 18–24 days a few up to 47 days In every case a solid tumour developed in this testis besides solid tumour nodules in the peritoneum and haemorrhagic ascites of up to 2 6 ml Transfers were made from the testis tumour to testis in other animals and from all animals with ascites such fluid was transferred to peritoneum of other animals After 3 ascites transfers most animals had only solid tumours or no reaction at all

After 8 testis testis transfers however one mouse developed 2 6 ml of bloody ascites with 1 per cent tumour cells in 18 days from which consecutive transfers of 0 6–1 8 ml ascites to the peritoneal cavity succeeded

Smears stained according to *Papanicolaou* and *Mowry* showed that the ascites from the 18th transfer contained 35 per cent tumour cells (Fig 6) From then on the ascites was considered a tumour and denoted MCGI AA (cf *Klein* 1955 a) It was further transferred by 0 4 0 8 ml intraperitoneally

Properties of the AA Form

Inoculum of 0 4 0 8 ml ascites tumour kills host in 10–16 days average 12 days and produces 5–10 ml ascites fluid with a tumour cell concentration of 10^7 13 10 cells/ml (Figs 7 8) In addition small solid tumour nodes were always found in the peritoneal wall Metastases to lungs or liver were not observed The critical inoculum dose of the AA tumour was estimated in adult mice after 27 transfers of the AA form Ascites tumour with statistically expected tumour cell concentration



Fig 8
MCGI AA 30th transfer generation (Papanicolaou × 1200)

from 10 down to 1 cell in 0 4 ml ascites fluid was injected to peritoneum of 3 mice for each cell dose Survival times and total tumour cell numbers are given in Table 1 Intravenously injected 50 000 cells 20 per cent takes after 18 days 10 000 cells 0 takes after 18 days

TABLE 1

Critical Intraperitoneal Inoculum Cell Dose of Ascites Tumour (AA)

Number of cells inoculated	Survival time in days mean (and individual mice)	Ultimate number of free tumour cells mean	Ultimate percentage of free tumour cells mean
10	13 (12 12 15)	3 2 10^8	86
10^6	16 (15 17 17)	2 0 10^8	92
10	20 (20 20 21)	1 8 10^8	89
10^4	26 (25 25 28)	2 9 10^8	99
10^3	32 (29 33 34)	3 0 10^8	95
10^{*}	37 (32 43)	3 1 10^8	90
10	50 (39 61 §)	1 8 10^8	86
1	35 (29 34 41)	2 1 10^8	84

* One animal with solid tumour only survival 81 days
§ One animal with solid tumour only survival 46 days

Injections of ascites tumour intraperitoneally were made with gauge no 12 throughout Some mice developed a small (up to 0 5 cm solid tumour in the ab dominal wall around the inoculation site but all these developed a typical ascites tumour too A few animals however developed a large solid tumour in the ab dominal wall only despite the fact that these inoculations most probably were given mainly intraperitoneally

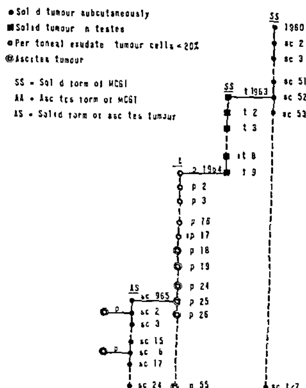


Fig 9
Propagation of MCC1 from Dec 1960 to March 1966

From the 14th transfer generation of the AA tumour 10^5 tumour cells were in oculated subcutaneously giving a solid tumour AS After 2 and 16 subcutaneous transfers a mince of AS inoculated to peritoneum reproduced the AA form (Fig 9) verifying the conversion to ascites form to be permanent (*Klein* 1955 b)

Properties of the AS Form

Subcutaneous inoculation 100 per cent takes Tumour palpable 3 days (pieces) kills host in 17 27 days Metastases to lungs (100 per cent 22 days) occasionally to lymph nodes never to liver Gross and m croscopic appearance similar to the SS form May 1965

TABLE 2

Transfer generation	Chromosome preparations: Number of metaphases	Chromosome preparations: Diploid cells in per cent	Papanicolaou smears Non tumourous cells in per cent
6	134	8	25
9	29	10	34
10	86	6	19
25	100	8	20

Chromosomal Investigation

Chromosome analyses of 9 days old ascites tumours (AA) were performed after Colcemid (Ciba) treatment according to *Hellström* (1959) agar fixation procedure

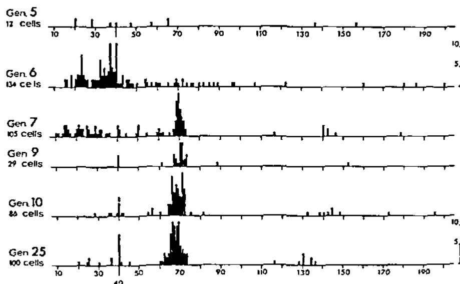
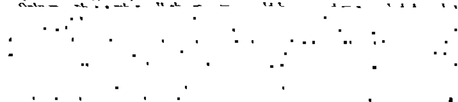


Fig 10

Distribution of chromosome numbers in 466 ascites cells at various transfer generations after establishment of an ascites tumour, MCGI AA Normal chromosome number of the mouse 2n = 40

modified after *Pacha & Kingsbury* (1962) and staining according to *Carr & Walker* (1961) Attempts were made to reduce the loss of chromosomes during preparation of materials by using the agar fixation method and no squashing

Photographs were taken with Zeiss photomicroscope using a planapochromatic oil immersion objective × 100 The chromosomes were examined on photographs (total magnification × 2500)



tions discussed by *Ford* (1964) seem negligible here

RESULTS OF CHROMOSOMAL INVESTIGATION

The first few generations of the tumour after conversion to ascites form were not studied Transfer generations 5, 6, 7 9 10 and, six months later, generation 25 were analysed A pronounced shift in the composition of the population appeared (Fig 10) The first generations studied showed an extensive variation in chromosome numbers with a hypodiploid modal region in the 6th generation Successive generations showed leaps in stemlines until a final clustering of 75 per cent of all cells in the hypotetraploid region was reached in the 25th generation This pattern of transformation with hypotetraploidy replacing hypo-

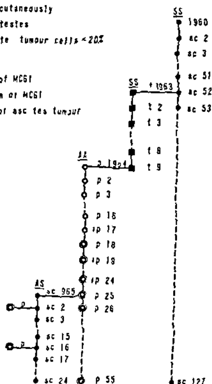


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Chromosome analyses of 9 days old ascites tumours (AA) were performed after Colcemid (Ciba) treatment according to *Hellström* (1959) agar fixation procedure

somes were observed As shown in Table 3 there was a relative increase of cells containing the large metacentric marker, parallel to the establishment of a hypotetraploid s-region The scatter in this modal region decreased and a concentration in the region of 66 to 69 has occurred while 71, which was the modal number in the 10th generation, essentially decreased in the 25th generation

TABLE 3

Transfer generation	Aneuploid cells with marker chromosome, in per cent	Cells in s region, in per cent	S cells with marker chromosome, in per cent	2s cells in per cent	Number of metaphases
6	23	25	18	7	134
7	61	37	88	6	105
10	88	70	92	7	86
25	92	75	99	7	100

Tumour cells with small numbers of chromosomes which were relatively frequent in the first generations studied are generally rare in ascites tumours in the mouse At the present time it cannot be conclusively determined whether these represent artefacts or the results of multipolar spindles that might possibly survive for a period in this special environment If the latter should be true, future experiments will be able to confirm this, and then more weight will have to be given to this observation It can also be further studied by means of cell cloning and cytochemical methods (*Bader* 1959, *Stich & Steele* 1962, cf *Mendelsohn* 1963) In rat ascites hepatoma, cells containing extremely few chromosomes have recently been reported (*Isaka* 1964)

It is not uncommon that ascites tumours lack an individual predominating modal number (*Isaka* 1964) Old ascites tumours are often hypotetraploid, as a rule karyotypically stable, and sometimes have markers (*Levan & Hauschka* 1953, *Ising* 1958, *Isaka* 1964) With regard to our tumour, however, a further progression or selection toward some, for the present, slowly growing clone would perhaps be expected All changes recorded hitherto can probably be explained by selection alone In addition rare endomitotic reduplications (*Levan & Hauschka* 1953) occur supplying the population with polyploid cells, as well as multipolar divisions, which might be examples of somatic reduction (*Rasch et al* 1959)

Our efforts to make complete karyotype analyses within the solid rhabdomyosarcoma have failed, so far The karyotypically interesting process of transformation to ascites tumour has thus been impossible to study It is known that primary methylcholantrene induced mouse sarcomas can have "new" chromosomes, for instance a large metacentric one, and extensive variation of chromosome numbers (*Hellström* 1959)

COMMENTS

The solid form, SS, of the tumour behaved like most transplanted tumours by changing its histological picture in the direction of cellular anaplasia, reduced host survival time and lack of metastases in animals with the shortest survival time (*Stewart et al* 1959, *Ostenfeld* 1941)

The reason for transformation to ascites form after repeated testis transfers is obscure The survival time for the animals with tumour in testis did not deviate from those given tumour mince to the peritoneum according to *Klein* (1951 a) Possibly, influence of hormonal milieu or hyaluronidase in testis could be responsible

The critical inoculum dose of ascites tumours, according to *Klein* (1951 b), is the lowest dose regularly producing ascites tumours and with survival time showing low variability, where solid growth is of minor extent and without increase of inflammatory cells These criteria were fulfilled by a dose of 10^3 cells Smaller cell doses gave ascites tumour in less than 100 per cent, highly variable survival times, but without increase of solid growth or inflammatory cells (Table 1) The inoculum dose of our ascites tumour seems low compared with most ascites tumours reviewed by *Klein* (1955 b)

It seems as though the development of ascites tumours was inhibited by solid growth in the abdominal wall This phenomenon will be investigated further Furthermore, additional tests of cell viability of SS AA and AS form of the tumour are in progress by intravenous and subcutaneous injections

The actions of coagulation and fibrinolysis on distribution, number and size of metastases after intravascular administration of cells from the solid and ascites forms of the tumour are reported in separate papers (*Boeryd* 1965, 1966)

SUMMARY

To provide an efficient tool for the study of metastases in an isologous tumour-host system, a rhabdomyosarcoma was induced by 20 methylcholantrene in CBA mice Morphological and biological properties of the tumour at various times were described The solid tumour which regularly gave spontaneous metastases was transformed to an ascites form Critical inoculum dose was found low for the solid as well as the ascites form Various transfer generations after the establishment of the ascites tumour were investigated cytogenetically Striking shifts in the distribution of chromosome numbers were shown with a final gathering in a hypotetraploid stemline region Almost every modal cell possessed one large metacentric marker chromosome

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EFFECT OF HEPARIN AND PLASMINOGEN INHIBITOR (EACA) ON INTRAVENOUSLY INJECTED ASCITES TUMOUR CELLS

By

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Cytological studies have revealed that in human subjects tumour cell aggregates are more frequent in local venous blood draining tumours and that solitary tumour cells predominate in peripherial blood (*Griffiths & Salsbury* 1965). This suggests that aggregated tumour cells are retained in the first capillary networks they reach. It remains, however, to be established whether aggregated tumour cells or single cells are most liable to establish metastases (*Coman et al.* 1951, *Coman* 1953, *Watanabe* 1954).

For the study of metastases tumour cell suspensions have been prepared from solid tumours by various mechanical means or with the aid of enzymes (*Madden & Burk* 1961, *Boeryd et al.* 1965). Whatever the dispersion method used, the tumour cells will be affected in some way, and it is unknown what this might do to the establishment of metastases.

Ascites tumours constitute suspensions of tumour cells, which may be unaffected by either mechanical or enzymatic factors. But other differences exist between the ascites tumour and the solid tumour from which it has been transformed. After intramuscular inoculation of the ascitesform, AS, and the solid form, SS, of the MCIM, both *Klein* (1955) and *Ringertz et al.* (1957) demonstrated greater metastasizing tendency to the lungs for the AS form than for the SS form. *Ringertz et al.* (1957) discovered also that, after intraperitoneal injection of these tumour forms, the SS form infiltrated into the peritoneum more readily than did the AS form. This was interpreted as an indication that in ascites tumours the adhesion between cells are reduced and that different cell types derived from the same original tumour varied with respect to the "soil" they prefered for their growth. Further *Purdom et al.* (1958) demonstrated that there was a progressive increase in

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negative electrical surface charge when the tumour passed from solid to ascites form

Comparing the metastasizing tendencies of intravenously injected mechanical suspensions of Walker carcinoma 256 and the ascites form of the same tumour, *Warren & Gates* (1936) found that metastases to the lungs appeared sooner and in greater numbers after injections of the ascites tumour but that the incidence of takes was similar after 10 to 25 days The ascites form of various tumours has subsequently been used by several workers for studying the effect of various factors on the establishment and growth of metastases from intravenously injected tumour cells (*Ambrus et al* 1956 *Selecki* 1959, *Clifton & Agostino* 1962, 1964 *Koike* 1964, *Wood* 1964 etc)

The aim of present investigations was to study how changes in coagulation (heparin) and in the fibrinolytic activity (epsilon amino caproic acid, EACA) affect the incidence size and distribution of metastases from an intravenously injected ascites tumour of MCGI, MCGI AA in isologous mice, and to compare the results with those of the corresponding solid tumour, MCGI SS (*Boeryd* 1965 1966)

MATERIAL AND METHODS

The experiments were performed on mice of the same strain as before (*Boeryd* 1965 1966) with the ascites form of MCGI (*Mellgren et al* 1966) the tumour being harvested on the 14th day after intraperitoneal transplantation Dilution to the required number of cells was done in modified Parker 199 (*Salk et al* 1954) The cells were counted in a haemacytometer Cell viability was estimated with Trypan blue 90 per cent of the tumour cells remained unstained The mice fed commercial pellets and water *ad libitum* were divided into 6 groups

Group I	18 mice pretreated with 0 2 ml saline intraperitoneally immediately prior to tumour cell inoculation
Group II	17 mice pretreated with 0 5 mg heparin (Vitrum Stockholm) in 0 2 ml solution intraperitoneally immediately prior to tumour cell inoculation
Group III	17 mice pretreated with 60 mg EACA (Kabi Stockholm) as 0 2 ml 30 per cent solution intraperitoneally immediately prior to tumour cell inoculation
Group IV	18 mice pretreated with 0 02 ml saline subcutaneously every 8 hours for 6 days
Group V	24 mice pretreated with 1 mg heparin in 0 02 ml solution subcutaneously every 8 hours for 6 days
Group VI	15 mice fed a powdered diet containing 30 per cent EACA for 6 days

Group I served as control for groups II III and VI and Group IV as control for group V

The actions of heparin and EACA were assayed as decribed previously (*Boeryd* 1965) The clotting time in mice given a single heparin dose exceeded 60 minutes after 5 hours and in mice treated with heparin for 6 days always exceeded 30 minutes for the entire period of treatment The lysis time of the coagulum exceeded 1 hour when tested after 2 hours it exceeded 30 minutes when tested after 5 hours from mice given a single EACA dose and from mice treated with EACA for 6 days always exceeded 30 minutes for the entire period of treatment The effect of single [illegible]

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and total volume of the metastases being estimated as described previously (*Boeryd et al* 1966) The significance of any differences between groups was determined with the aid of Wilcoxon s two sample rank test

Differences with $P < 0.05$ were accepted as significant

RESULTS

At autopsy small gross metastases were found to the lungs otherwise no gross metastases were encountered

After a single heparin dose the number of metastases to the lungs was not affected but their average volume tended to be smaller and their total volume was reduced as compared with the corresponding controls Continuous heparin treatment for 6 days increased the num ber of metastases to the lungs but reduced their average and total volumes compared with the corresponding controls (Table 1)

TABLE 1

Metastases to Lungs All Mice with or without Metastases Individually

Group		Incidence of takes	N	$v \times 10^{6}$	$V \times 10^{3}$
I	Controls	18 18	432	14	5
II	Heparin	15 17	476	4	2
III	FACA	14 17	188	17	3
IV	Controls	17/18	363	26	9
V	Heparin	17 18	1091	2	2
VI	FACA	14 15	281	32	9

After EACA in single dose or prolonged administration the number of metastases to the lungs seemed reduced and the average volume seemed increased but owing to the skew distribution of the data no differences not even on the $P = 0.1$ level (Table 1)

These results of heparin and EACA treatment were obtained whether all mice or just those with metastases were compared with the relevant controls

Histological examination of the liver disclosed no metastases in groups I and II In groups III IV V and VI the number of mice with few small metastases was 3 3 6 and 1 respectively

DISCUSSION

The present investigation disclosed considerable differences in meta stasizability after intravenous inoculation between the ascites and solid forms of MCGl In control mice—in spite of a higher cell dose—the ascites tumour gave rise only to metastases to the lungs while the solid tumour produced numerous extrapulmonary metastases (*Boeryd* 1965, 1966)

The two tumour forms showed marked differences in their responses to heparin and EACA as well (cf. *Boeryd* 1965, 1966)

Thus single heparin dose had no effect on the incidence of extrapulmonary takes nor on the number of pulmonary metastases from MCGI-AA A similar heparin dose reduced the number of metastases to the lungs and increased that to the liver from MCGI SS (*Boeryd* 1965) Furthermore such a heparin dose tended to reduce the average volume and significantly reduced the total volume of metastases to the lungs from MCGI-AA, but tended to exert the opposite effect on mice inoculated with MCGI-SS

Prolonged administration of heparin for 6 days had no effect on the incidence of extrapulmonary takes from MCGI-AA but tended to increase the incidence of subcutaneous takes and to reduce those in the lymph nodes from MCGI-SS (*Boeryd* 1966) Similar treatment increased the number and reduced the average volume and total volume of metastases to the lungs from MCGI-AA, the number of pulmonary metastases from MCGI-SS increased but their average volume tended to diminish and their total volume was reduced at the same time as the average volume and total volume of such metastases to the liver were increased

EACA, whether given as a single EACA dose or for 6 days, had no significant effect on MCGI AA metastases A single EACA dose, on the other hand, increased the average and total volumes of pulmonary metastases from MCGI-SS (*Boeryd* 1965) After prolonged EACA administration the corresponding factors showed almost significant increases (*Boeryd* 1966)

Thus, whereas single or repeated heparin doses on the whole benefitted transpulmonary passage of intravenously inoculated MCGI SS cells and EACA seemed to inhibit such passage, no similar manifestations were demonstrated in the case of MCGI-AA

A variety of reasons for these differences in metastasizability suggest themselves *Ringertz et al* (1957) postulated that the biological properties of a tumour's ascites form may deviate from those of its solid form, the cells of the former showing a preference for establishing metastases to the lungs Moreover, trypsination might affect the MCGI *SS cells to* grow in certain organs (*This problem is at present being* studied) A third possibility would be that the ascites tumour cells might be better dispersed in the blood than the MCGI-SS cells after enzymatic treatment, and that single tumour cells could be less capable of establishing metastases than small cell aggregations

The effect of heparin on the average size of pulmonary metastases might be due to reduced thrombus formation around the tumour cells But if so EACA probably should have exerted an opposite action Actually, however, EACA had no effect whatsoever on the growth of ascites tumour metastases Conversely heparin could exert its action by inhibiting aggregation of the tumour cells, which would make the

metastases smaller and more numerous, as was the case after prolonged treatment with heparin It is difficult to explain how heparin could exert a cytotoxic inhibition on the growth of metastases, because then the number of metastases should not increase, nor has any such inhibition been demonstrated *in vitro* (*Lisnell & Mellgren* 1963)

SUMMARY

Ascites tumour, MCGI AA transformed from a 20 methylcholantrene induced rhabdomyosarcoma, MCGI, was inoculated intravenously in isologous CBA mice treated with heparin or EACA in single doses or continuously for 6 days The results were compared with those of the same experiments with the corresponding solid tumour, MCGI SS Whereas single or repeated heparin doses on the whole benefitted transpulmonary passage of intravenously inoculated MCGI SS cells and EACA seemed to inhibit such passage, no similar manifestations were demonstrated with MCGI-AA The reasons for these differences were discussed

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THE VASCULAR REACTION IN THE FREE SKIN ALLO- AND AUTOGRAFTS

A Stereomicro-Angiographic and Histological Study in the Rabbit

By

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Received 15 ii 66

It has been shown that 2 to 3 days after transplantation the free skin allograft has a vascular supply connected to the vasculature of the surrounding host tissue (*Scothorne & McGregor* 1953, *Taylor & Lehrfeld* 1955, *Converse et al* 1958, *Lambert et al* 1965) The stereoscopic appearance of the microvasculature of the graft, and the changes that this vascular pattern undergoes during the rejection and ultimate necrosis of the graft have, however, not been assessed

In the present investigation the vascular patterns in free skin allo- and autografts were studied by a combined stereomicro-angiographic and histological method at various intervals after transplantation

MATERIAL AND METHODS

Sixteen female albino rabbits from different litters and weighing between 3 0 and 4 0 kg were used They were maintained on standard laboratory pellets and tap water

The rabbits were grouped in pairs each rabbit serving both as donor and recipient for its partner One full thickness circular skin graft 2 cm in diameter was taken from the back of each ear The epichondrial tissue was left intact The graft from the right ear was used as an autograft and placed on the defect on the left ear The graft from the left ear was used as an allograft and transplanted to the defect on the right ear of the partner The operations were performed with the animals under pentobarbital anaesthesia supplemented with ether

In six rabbits the grafts were taken from the midline of the distal half of the ear and included the large central artery which was ligated on the ear side and left open in the transplant. In the ten remaining rabbits the grafts were taken from the distal half of the ear between the central and marginal vessels so that no large vessels were ligated or included in the grafts The grafts were held in position by uninterrupted sutures and left uncovered for daily inspections

The animals were sacrificed at various intervals from the 1st to the 10th day after transplantation and the grafts examined Some animals were sacrificed when the allograft showed gross signs of necrosis, other rabbits were sacrificed prior to the development of any such changes

The grafts were examined by the stereomicro-angiographic method (*Bellman* 1953) as modified by [illegible] *et al* (1963 for combined stereomicro-angiographic and histological *examination of surgical and autopsy specimens* For this a cannula was inserted in the main artery at the base of the ear and a 75 per cent aqueous suspension of barium sulphate (Micropaque) injected. When no more filling of the

finest ear vessels was grossly seen and contrast medium returned via the opened vein at the ear base, the injection was stopped, the artery ligated and the ear fixed in 10 per cent neutral formalin for 3 days The graft with a 1 cm surrounding zone of ear was embedded in a mixture of wax and paraffin and 200 to 1200 micron thick blocks cut These were stereomicro angiographed in a special X ray tube (Machlett OEG 50) using a high resolution photographic emulsion (Kodak Maximum Resolution Plates) The micro angiographed blocks were re embedded in paraffin and sections cut for histological examinations Haematoxylin eosin van Gieson s stain alone or counterstained for elastic fibres with Weigert's stain, and Ladewig s stain for fibrinoid substance were used

RESULTS

The morphological pictures varied considerably with the intervals between grafting and injection Some variations in the morphology were noted also between different grafts injected after the same interval, probably depending on variations in the immunological response between the different rabbits The variations with time were, however, dominating

1 3 Days after Transplantation

Six animals were sacrificed within the first three days following the transplantations In one of these six rabbits the filling of contrast medium failed for both ears In the remaining five rabbits the injections were successful All the grafts were viable

Allografts In one of the grafts, removed two days after transplantation, occasional large vessels were visualized in the graft close to the margin (Fig 1 A) All the other grafts were entirely avascular in the micro-angiograms

The grafts were oedematous and haemorrhagic There was mild or moderate infiltration by polymorphonuclear cells Fibrin thrombi were seen in some wide and thin-walled vessels There was evidence of extra vascular precipitation of fibrin in the oedematous and haemorrhagic areas, particularly along the base of the graft Along the base and the margin of the graft there was slight evidence of the formation of granulation tissue with fibroblasts and capillary channels, none of which were contrast filled The contrast-filled vessels in one of the grafts were wide and thick-walled (Fig 1 B)

Autografts In none of the autografts was there any evidence of vascularization in the micro-angiograms The grafts were oedematous and haemorrhagic Some thrombi were seen in small vessels Infiltration by inflammatory cells was minimal

4 Days after Transplantation

Three rabbits were sacrificed 4 days after the transplantation In one of them the allograft appeared necrotic In the other two rabbits there was no difference in gross appearance between the allografts and autografts



Fig 1

A Micro angiogram from an allograft 2 days after transplantation Suture line along the lower edge of picture Both wide and thin vessels are seen in the graft The transected wide vessel in top of figure is the pre existing large central artery in the graft × 30

B Histological section from the area depicted in Fig 1 A The graft is oedematous One artery is completely filled with contrast medium (larger arrow) another artery with a modified thickened wall contains small aggregates of contrast medium (smaller arrow) Haematoxylin eosin × 170

Allografts The grossly necrotic allograft was micro angiographically avascular and in the histological sections the vessels were collapsed and the tissue necrotic In the remaining two allografts irregular vessels with varying calibres were seen along the margin and at the base of the grafts (Fig 2 A) The marginal vessels derived from vessels in the surrounding ear tissue and the vessels along the base from the underlying epichondrial tissue The vessels were kinked slightly tortuous and of arteriolar and capillary calibre These vessels had thin walls like capillaries Numerous interconnections were seen between these vessels In one of the allografts some wide vessels were visualized in the central portion of the graft these were capillaries and either thin walled arteries or wide arterioles

The vascular pattern of the graft was quite different from the loose and regular network of straight or curved delicate vessels seen in the surrounding normal ear tissue (Fig 2 A)

The two grafts with evidence of vascularization showed oedema and

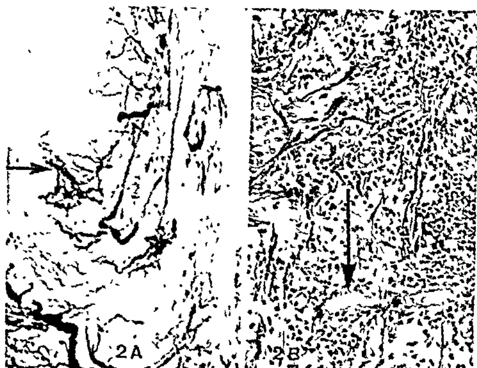


Fig 2

A Micro-angiogram including the graft-host junction (arrow) of an allograft 4 days after transplantation Above arrow, host tissue vascularized by a regular network of mainly delicate vessels Below arrow graft vascularized by irregularly coursing vessels of varying calibres, most evident in lower left corner of picture × 30

B Histological section from the area depicted in Fig 2 A The graft is oedematous and infiltrated by mononuclear and some polymorphonuclear cells A contrast filled vessel is seen at arrow van Gieson Elastic × 170

haemorrhages and a moderate infiltration by inflammatory cells Multiple small thrombi were seen and some vessels showed fibrinoid changes in their walls In the marginal and basal areas of vascularization there was a loose connective tissue rich in fibroblasts (Fig 2 B)

Autografts In the micro-angiograms there was evidence of vascularization along the margins and bases of the grafts, similar to that seen in the allografts There was a moderate degree of oedema and haemorrhage as well as thrombus formation Inflammatory cells were seen in moderate numbers throughout the grafts

7 Days after Transplantation

Four rabbits were sacrificed 7 days after the transplantation In one of them the allograft showed a deep brown discoloration in a circumscribed area occupying about 15 per cent of the surface The remaining portion of this graft and the three other allografts were slightly darkened, whereas the autografts appeared grossly unaltered



Fig 3

A Micro angiogram of an *allograft* 7 days after *transplantation* The graft is vascularized from the *epichondrial* layer (right) by *tortuous* vessels which run perpendicular *towards* the surface (left) × 30

B Histological section *from the* area depicted in *Fig* 3 A showing the pre existing large central *artery* in the graft This contains a *thrombus* and is invaded by fibrous tissue which *contains* fragments of elastic *lamellae* There is some contrast medium *peripherally* in the lumen (left) The wall of the artery is disintergrating van Gieson Elastic × 65

Allografts In two of the grafts there was an apparently complete vascularization whereas in the other two avascular areas alternated with areas of visualized vessels The vascular pattern consisted of vessels of arteriolar calibre which derived mainly from the underlying epichondrial layer and ran towards the surface of the graft in tortuous courses (Fig 3 A) Along their courses these vessels gave off tortuous capillaries which formed dense networks In the superficial layer the vessels split up to form similar capillary networks Inside the graft a number of wide vessels were visualized which had courses parallel to the surface

Histologically two of the grafts showed large irregular areas of necrosis whereas the two fully vascularized grafts were necrotic only in small superficial areas The vessels ran from the epichondrial layer to the surface of the graft in a rather loose fibrous tissue rich in fibroblasts There was a dense infiltration of cells most of which were *mononuclear* The vessel walls were thin of pre capillary or capillary type some with fibrinoid staining properti Th mi ro angiographically

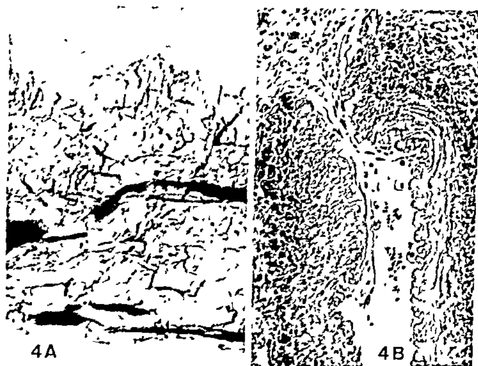


Fig 4

A Micro angiogram of an autograft 7 days after transplantation Bottom the cartilage plate The graft is vascularized by a network of precapillary and capillary vessels A few wide arteries are seen in the graft × 30

B Histological section from the tissue depicted in Fig 4 A, showing a pre existing thick walled graft artery The wall of the artery is destroyed (top left) and invaded by a connective tissue rich in fibroblasts Ladewig × 170

visualized, wider vessels that ran parallel to the surface were modified arterioles and arteries, including the large central artery in one graft The wall of this artery was disintegrating with fragmentation of the elastic lamellae (Fig 3 B). The lumen contained contrast medium and a thrombus which was invaded by fibroblasts and contrast filled capillaries

Autografts All four grafts showed complete vascularization (Fig 4 A) The pattern consisted of pre-capillary vessels which derived from the epichondrial layer and ran towards the surface of the graft The vessels appeared thinner and less tortuous than in the allografts The vessels gave off capillaries which ran at right angles through the graft and formed loose networks Occasional wide vessels were visualized

Histologically there was an abundant, vascular connective tissue sparsely infiltrated by polymorphonuclear and mononuclear cells Connective tissue was seen to invade large arteries as in the allografts (Fig. 4 B), and contrast medium was seen in the lumina of some of these arteries

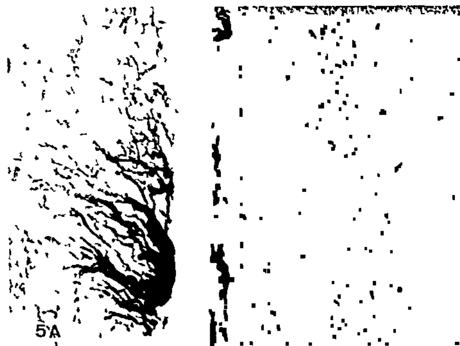


Fig 5

A Micro angiogram of an allograft 10 days after transplantation Right cartilage plate Vascularization is evident in the basal layer of the graft In bottom half of picture the vessels are seen to arise from the epichondrial layer and invade the graft perpendicularly There are wide variations in vessel calibre Part of the graft is avascular (left) × 30

B Histological section of the area depicted in Fig 5 A Right the viable basal layer of the graft containing contrast filled vessels Left necrosis corresponding to the avascular area in Fig 5 A Haematoxylin eosin × 65

10 Days after Transplantation

Three rabbits were sacrificed 10 days after the transplantation Injection was successful in all of the ears except in one ear with an autograft

Allografts One of the allografts was avascular and showed complete necrosis The other two allografts were partially vascularized in that wide branches were seen along the margins and at the base of the graft from which regions vessels of varying calibres ran towards the inner portion of the graft (Fig 5 A) There wide avascular areas were seen In one of the grafts however some vessels were also seen in the central portion The superficial layer was avascular in both grafts

Histologically there was proliferating fibrous tissue with capillaries along the margin and the base of the grafts In the central and superficial areas there was necrosis (Fig 5 B) In the graft with some vessels in the central portion these occurred in viable tissue between patches of necrosis Some thrombi were seen in thin walled and wide vessels

The large central artery was included in one graft, it was invaded by a vascularized connective tissue There was a moderate infiltration of mononuclear cells throughout the grafts

Autografts Complete vascularization was seen in both successfully injected grafts The vascular pattern was not appreciably different from that seen in 7-day-old autografts

In the graft tissue there was fibrosis, proliferation of capillaries and some cell infiltration There was also evidence of replacement of large graft vessels by a vascularized connective tissue

DISCUSSION

There is general agreement that the free skin allo- and autografts are nourished by an avascular "plasmatic" circulation during the first 2 to 3 days following grafting After this phase, circulation through vessels is slowly re-assumed in the graft, as shown by *in vivo* injections of diffusible stains (*Scothorne & McGregor* 1953) and direct stereomicroscopy (*Taylor & Lehrfeld* 1955)

Injections with Indian ink (*Scothorne & McGregor* 1953) and radiopaque material (*Lambert et al* 1965) have clearly shown that, when circulation is re-established, the vasculature in the graft communicates with that of the surrounding host tissue It has been claimed that this communication is established by the formation of anastomoses between the original vasculature of the graft and the surrounding host vessels (*Taylor & Lehrfeld* 1955) There is, however, histological (*Medawar* 1944, *Converse et al* 1958) and histochemical (*Converse & Ballantyne* 1962) evidence that, in the skin graft, the original vessels are replaced by an ingrowing, newly formed vasculature

The findings in the present investigation confirm that after an initial avascular phase both the allo- and autograft become vascularized by the ingrowth of new vessels from the surrounding host tissue These vessels were found to derive mainly from the bed underlying the graft and to transverse the graft perpendicularly towards the surface in spiral courses This vascular pattern was quite different from that seen in the surrounding host tissue

The fact that contrast medium was seen in the easily identified pre-existing arteries in the graft indicates that contact was also established between the vasculature of the surrounding host tissue and the original graft vessels Many of these vessels were, however, thrombosed with disintegrating walls, and displayed evidence of being replaced by a vascularized connective tissue The findings indicate that, although connection may be established between the vasculature of the host tissue and the original graft vessels, these connections are of minor importance and probably only temporary, the graft being mainly supplied by a newly formed vascular network

No significant differences were observed between the vascular pat

terns of the allografts and autografts unless necrosis was present in the allografts. This is in agreement with observations on the re-establishment of the circulation in the two types of grafts studied *in vivo* (*Scothorne & McGregor* 1953, *Taylor & Lehrfeld* 1955, *Lambert et al.* 1965) and indicates that the rejection and development of necrosis in the allograft is not primarily due to a faulty development of the vascularization of the graft as suggested by *Conway et al.* (1952).

Whether some alterations in the established vasculature of the allograft may appear and cause necrosis cannot be determined from the findings in the present investigation. The absence of visualized vessels in the necrotic areas cannot be ascribed any pathogenic significance, since it may be a feature secondary to the necrosis. It has been shown that in the transplanted kidney the vascular alterations accompanying the graft reaction can lead to the formation of pathological arteriovenous communications, which may be significant in the progressive destruction of the graft (*Almgård et al.*). The transplanted kidney has well defined arterial and venous systems, however, and this makes vascular alterations and interconnections easy to identify and evaluate. In the free skin allograft, which is supplied by newly formed pre-capillary and capillary vessels, the inflow and outflow vascular systems are difficult to identify in post mortem specimens and any pathological communications between vessels can hardly be assessed, since the occurrence of intervascular communications is a normal feature at this level in the vascular tree.

SUMMARY

The vascular patterns of free skin allo- and autografts in the rabbit were studied at various intervals after transplantation by a combination of stereomicro-angiographic and histological techniques.

The grafts were vascularized by the ingrowth of pre-capillary and capillary vessels, first evident 2 days after transplantation. The ingrowing vessels derived mainly from the underlying graft bed, and described spiral courses in the graft.

Contrast medium was also seen in the original graft vessels. These vessels displayed degenerative changes with thrombosis and evidence of being replaced by a vascularized connective tissue.

No significant differences between the vascular patterns in allo- and autografts were noted unless the allograft displayed necrosis. The necrotic areas were avascular.

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THE EFFECT OF THE HISTAMINE LIBERATOR, COMPOUND 48/80 ON MAST CELLS IN NORMAL PERIPHERAL NERVES

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The distribution of mast cells in different compartments of normal peripheral nerve trunks has been outlined in previous investigations in the rat (*Enerback et al* 1965, *Olsson* 1965) Numerous mast cells were found in the epineurial and perineurial sheaths of the sciatic nerve and its main branches They were also present in the endoneurium in the interstices between the nerve fibres

Mast cells are known to contain biologically highly active substances Such cells isolated from rat peritoneal fluid have been shown to contain heparin, histamine, 5 hydroxytryptamine (serotonin 5 HT) and an alcaline proteolytic enzyme (for ref see *Padaver* 1957, 1963, *Riley* 1959, *Smith* 1963, *Bloom* 1965)

Mast cells are highly fragile and responsive cells Exposure to a large variety of stimuli may result in a release of biogenic amines and concomitantly a series of structural cellular changes, *e g*, formation of cytoplasmic vacuoles and discharge of granules This process is usually termed degranulation of mast cells One of the most potent and in experimental work most widely used histamine liberators is a condensation product of paramethoxyphenethylmethylamine and formaldehyde called Compound 48/80 (*Baltzy et al* 1949)

Systemic administration of Compound 48/80 is followed by degranulation of mast cells in most tissues of the rat (*Mota et al* 1953, *Norton* 1954, *Fawcett* 1954 1955, *Riley & West* 1955, *Smith* 1963, *Singleton & Clark* 1965) Recently however, *Enerbäck* (1966 a) observed that mast cells in rat gastrointestinal mucosa differ from mast cells in the skin in being insensitive to Compound 48/80 A selective insensitivity of mast cells to Compound 48/80 does not seem to be restricted to the intestinal mucosa Thus it has earlier been noticed that mast cells associated with small peripheral nerve branches in the skin of the rat also remain morphologically unchanged after intraperitoneal injections of Com

Compound 48/80 was kindly supplied by The Wellcome Research Laboratories Beckenham Kent England

pound 48 80 (*Riley & West* 1955, *West* 1957) However, the general occurrence of resistant mast cells in peripheral nerves has not been proved and their topographical relation to various compartments of the peripheral nerves has not been investigated

The aim of the present study is to outline the reaction pattern of mast cells in different parts of the peripheral nervous system and in different compartments of peripheral nerve trunks to the intraperitoneal administration of Compound 48 80 In addition, the cause of the *resistance of neural mast cells to Compound 48 80* is elucidated by giving microinjections of the compound into or by direct application of it around the sciatic nerve

MATERIALS AND METHODS

Sprague Dawley rats of both sexes were used They weighed between 150 and 225 grams at the beginning of the experiments The animals were kept on a diet of commercial rat pellets and water ad libitum Compound 48/80 was dissolved for injection in Ringer solution Groups of three to five rats were killed by decapitation at suitable intervals after injection and pieces of tissue were taken for histological examination

Intraperitoneal injections Three experimental groups received different dosage schedules using modifications of *Riley's* (1959) scheme The first group was given an intraperitoneal injection of 200 micrograms of Compound 48/80 per 100 gram

the rats on the second group for the first three days followed by daily injections of 500 micrograms of the drug per 100 gram of body weight

The animals were sacrified not later than thirty minutes after the last injection and the two sciatic nerves were taken for histological examination In some of the experimental animals and untreated control rats the following parts were als examined dorsal and ventral spinal nerve roots dorsal root ganglions sciatic plexus common peroneal nerve tibial nerve sural nerve brachial plexus and ear

Microinjections into nerves The injections were performed with a very fine glass capillary connected via a short plastic tube to a micrometer syringe[1] This instrument allows injections of very small volumes at low speed The right sciatic nerve was exposed high up in the thigh under ether anesthesia Under a dissection micr scope the glass capillary was inserted distally through the sheaths (epineurium and perineurium) into the nerve at about 45° to its long axis Care was taken to avoid teasing the nerve during the injection

In control experiments varying volumes of Ringer solution were injected after which the morphological changes of neural mast cells and nerve fibres were studied In the main control series 0 005 ml Ringer solution was injected into the nerve and the rats were studied six hours after the injection

In the experimental series 10 micrograms Compound 48 80 in 0 005 ml Ringer solution was injected into the nerve and the rats were allowed to survive for as long as those in the control group

Numerous investigators have demonstrated that after injections into nerves the fluid is transported for long distances along the nerves (*Weiss et al* 1945 *French Strain & Jones* 1948 *Brierly & Field* 1949 *Dorany & Matzke* 1960) Though the exact pathway for the spread of fluids within nerves is still unknown one principal way is probably through the extracellular space in the endoneurium A substantial extra cellular space in the endoneurium of peripheral nerves has recently been demonstrated in an electronmicroscopic investigation by *Thomas* (1963)

Direct application to the nerve sheaths of exposed nerves The right sciatic nerve was exposed under ether anesthesia About 0 5 ml Ringer solution containing 100

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1 Agla Micrometer Syringe Outfit Burroughs Wellcome and Company London

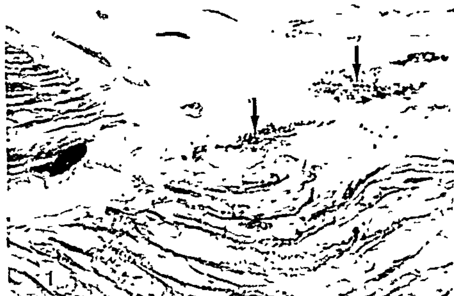


Fig 1

Mast cells in the sciatic nerve of a rat after a single intraperitoneal injection of Compound 48 80 Mast cells in the epineurium and the perineurium are degranulated (arrows) in contrast to the mast cell in the endoneurium Toluidine blue 920×

[illegible]

(*Enerbäck* 1966 b), which has proved suitable for studies of mast cells in peripheral nerves (*Enerback Olsson & Sourander* 1965) The two sciatic nerves were embedded in the same paraffin block Paraffin sections 5–7 microns thick, were stained in a 0 5 per cent aqueous solution of toluidine blue in a citric acid disodium phosphate buffer according to *Mellwain* with pH adjusted to 4 5 The sections were rapidly taken through alcohol to xylol and mounted in canada balsam

Myelin sheaths were stained with the luxol fast blue cresyl violet method (*Kluver & Barrera* 1953), while Palmgren's silver method was used for axons (*Palmgren* 1948)

Quantitative determinations Endoneurial mast cells were counted in adjacent visual fields of longitudinal sections of the sciatic nerve in the experiments with microinjections into the nerve In each rat about 200 mast cells were counted and classified as normal or degranulated The criterion for degranulation was the presence of granules outside the cell The percentage degranulation of endoneurial mast cells was calculated

RESULTS

The first intraperitoneal injection of Compound 48 80 induced signs of pruritus, salivation lachrymation and cyanosis of ears and nasal area, followed by edema in the skin These reactions were much less pronounced after subsequent injections, becoming minimal or entirely absent after three to four days Local injections of Compound 48 80 into the nerves did not produce such strong general signs

Intraperitoneal injections of Compound 48 80 Rats studied thirty



Fig 2.
Morphologically unchanged endoneurial mast cell of the sciatic nerve after intra peritoneal injections of Compound 48/80 for fourteen days 190 × a Phase contrast micrograph showing the close connection of the cell to the perineurium b Toluidine blue



Fig 3
Resistant endoneurial mast cells close to endoneurial blood vessels after intraperitoneal injections of Compound 48 80 for fourten days Toluidine blue 210 ×

minutes after a large single intraperitoneal injection of Compound 48/80 displayed a heavily disruption of epineurial and perineurial mast cells, with scattering of cytoplasmic granules around the cells (Fig 1) Some cells exhibited only minimal discharge of granules, while a few showed no clear change at all Injections of increasing doses of Compound 48 80 for three and fourteen days were followed by almost complete depletion of epineurial and perineurial mast cells These mast changes were observed at all levels of the peripheral nervous system examined, *e g* scitatic plexus, sciatic nerve, common peroneal nerve, tibial nerve sural nerve and brachial plexus, as well as in mast cells of and outside the capsule of, dorsal root ganglions



Figs 4 a and b
Degranulated endoneurial mast cells after microinjections of Compound 48 80 into the endoneurium of sciatic nerves Toluidine blue 500 ×

In contrast to the changes of mast cells in the epineurium and the perineurium no animals displayed morphological changes of mast cells in the endoneurium Resistant endoneurial mast cells were observed after a large single intraperitoneal dose as well as after daily injections for up to fourteen days (Fig 1, 2, 3) Unchanged endoneurial mast cells were found at all levels of the peripheral nervous system with the exception of dorsal and ventral spinal nerve roots and the dorsal root ganglions where no mast cells were found in nontreated control rats Within the endoneurium of the peripheral nerves, morphologically unchanged mast cells were seen in all areas, even close to the perineurium (Fig 2), and sometimes even close to endoneurial blood vessels (Fig 3)

Tissue from the ears displayed the same mast cell reactions as in the epineurium, *e g* a rapid discharge of granules from the cells and, after prolonged administration of the drug, depletion of mast cells An exception was the endoneurium of small peripheral nerve branches, in which the mast cells remained intact

Microinjections into nerves In the experimental series with injection of Ringer solution and Compound 48 80, the volume of 0 005 ml was chosen because of the volumes tested it elicited the minimal microscopical changes of both mast cells and nerve fibres

After injection of Ringer solution into the sciatic nerve most endoneurial mast cells appeared normal However the site of injection presented a few degranulated mast cells both in the endoneurium and in the epineurium Mast cells in the rest of the sciatic nerve appeared normal

Injections of Compound 48 80 were followed by marked mast cell changes not only at the site of injection but also along the whole length of the sciatic nerve (Fig 4) The majority of the endoneurial

mast cells was degranulated with scattering of metachromatic granules into the interstices between the nerve fibres. Only a few mast cells remained morphologically unchanged in the most distal part of the sciatic nerve. Such advanced mast cell changes were also observed in the epineurium and the perineurium most probably due to leakage of Compound 48/80 from the injection site.

Endoneurial mast cells were counted below the injection site of about 1.5 cm long longitudinal sections of the sciatic nerve. The proportion of degranulated cells among mast cells counted consecutively was determined after the injection of Ringer solution with and without Compound 48/80. The results are given in Table 1. As the table shows there is a very marked difference between the percentage of degranulated endoneurial mast cells after the injection of Ringer solution with and without Compound 48/80.

TABLE 1

Percentage of Degranulated Endoneurial Mast Cells after Microinjection of Ringer Solution with and without Compound 48/80 into the Sciatic Nerve of Ten Rats

Ringer solution alone	3.9	4.4	8.3	8.8	12.3
Compound 48/80 in Ringer solution	73.4	76.5	79.0	88.0	93.0

Direct application of Compound 48/80 to the sheaths of exposed sciatic nerves. Marked morphological changes occurred in epineurial and perineurial mast cells following the direct application of Compound 48/80 for five or thirty minutes. All rats displayed extensive disruption of these mast cells with scattering of granules from the cytoplasm (Fig. 5). Mast cells in the endoneurium, on the other hand, remained morphologically unchanged (Fig. 5). Intact endoneurial mast cells were frequently observed adjacent to the perineurium in both experimental groups.

DISCUSSION

The present study demonstrates that mast cells in he endoneurium and the epineurium of peripheral nerve trunks differ in their response to intraperitoneal injections of Compound 48/80. Thus endoneurial mast cells remain morphologically unchanged, while epineurial mast cells degranulate as most other mast cells in the rat.

Results of experiments designed to elucidate the cause of the resistance of endoneurial mast cells in peripheral nerves to intraperitoneal injections of Compound 48/80 have not previously been published. These cells may differ qualitatively from other mast cells in the rat. Another explanation could be that the endoneurial mast cells are insulated in some way from the action of the histamine liberator.

The mechanism by which Compound 48/80 brings about degranulation of rat mast cells and release of their biogenic amines has been the subject of many studies (for ref. see *Paton* 1957, *Green* 1962, *Uvnäs*



Fig 5

Degranulated mast cells in the perineur um and the epineurium (arrows) and intact end neurial mast cell (left) after local application of C mpound 48 80 to the surface of exposed sciatic n rves 490 × a Phase contrast micrograph showing the anatomical location of the mast cells b Toluid ne blue of the same field

1964, *Westerholm* 1964) Though the details of this mechanism are not yet clear, it has been shown to be complex and to depend on enzymatic processes (*Uvnas* 1964, *Westerholm* 1964).

Mast cells degranulate rapidly when Compound 48/80 is brought into contact with them *in vitro* (*Lagunoff & Benditt* 1959, *Uvnas & Thon* 1959) When this histamine liberator was directly injected into the endoneurium of the sciatic nerve in the present study, the endoneurial mast cells degranulated for a long distance along the nerves, while in the control experiments injections of Ringer solution alone produced local mast cell changes only These findings strongly indicate that the different response of endoneurial and epineurial mast cells to intraperitoneal injections of Compound 48/80 is not due to cellular differences but to the fact that the histamine liberator is prevented from reaching the endoneurial mast cells via the blood

The sheaths surrounding peripheral nerve fasciculi have been subjected to extensive physiologic and morphologic studies and it has been *shown that many inorganic ions and several other substances, when* applied to the surface of peripheral nerves, pass through the sheaths only with difficulty or not at all (for ref see *Lehman* 1957, *Martin* 1964) The location usually suggested for this diffusion barrier is the perineurial epithelium, a conclusion based in most cases upon indirect evidence from comparisons between normal and desheathed nerves

The perineurial epithelium consists of multiple laminas of cells, which form continuous circumferential coverings around the individual nerve fasciculi (*Shanthaveerappa & Bourne* 1962, 1963, *Thomas* 1963, *Martin* 1964, *Gamble* 1964 *Waggener et al* 1965) The laminas are built up out of closely apposed cells, which are supported by a basal membrane (*Rohlich & Knoop* 1961, *Waggener et al* 1965) From the morphological point of view the perineurial epithelium seems well equipped to restrict the flow of substances The present finding of degranulated mast cells in the innermost portions of the nerve sheaths and preserved endoneurial mast cells in the outer parts of the endoneurium after intraperitoneal injection suggests that a diffusion barrier to Compound 48 80 is located in the perineurium

A *diffusion barrier in the perineurium is not a sufficient explanation* for the observed resistance of the endoneurial mast cells to intraperitoneal injections of Compound 48 80 In addition one must assume a structure within the nerve fasciculi with the capacity of preventing access to the endoneurial mast cells from the endoneurial blood vessels

Little is known at present about the factors regulating the exchange of various substances between the endoneurial blood vessels and other structures in the peripheral nerves However, recent studies have shown that certain dyes and proteins injected intravenously in laboratory animals do not pass from the blood into the nerve parenchyma, whereas in other tissues they do reappear in extravascular sites (*Waksman* 1961)

The structure that obstructed the transfer of Compound 48/80 from the blood in the present study obviously must be localized between the lumen of the endoneurial blood vessels and the surface of the endoneurial mast cells. The results, however, cannot provide detailed information about the structure to which this function is associated, partly because the ultrastructural relationships between mast cells and blood vessels in the endoneurium have not been clarified. The observation of preserved mast cells in close association with endoneurial blood vessels suggest that the obstacle is presented by the vascular walls themselves. This hypothesis is supported by the finding of degranulated endoneurial mast cells after microinjections of Compound 48/80, indicating that when outside the endoneurial blood vessels this compound is not prevented from reaching the surface of the endoneurial mast cells.

Mast cells in peripheral nerves of the rat most probably have the capacity to synthesize and store histamine. Thus it has been established that peripheral nerves contain histamine (*Kwiatkowsky* 1943, *Euler* 1956, *Werle* 1956, *Werle & Schauer* 1956, *West* 1957, *Green* 1964) and histidine decarboxylase (*Holz & Westermann* 1956, *Werle* 1956). Within the peripheral nerves there seems to a correlation between the distribution of mast cells and histamine. Both are present along the entire length of the nerve trunks; removal of the sheaths, which normally contain a lot of mast cells, decreases the content of histamine (*Rexed & Euler* 1951). In addition a rough correlation is found between the histamine content and the number of mast cells in comparison of different nerves from various animal species (*Torp* 1961).

It has been reported that most of the histamine in the sciatic nerve of the cat is not released after intra-arterial infusions of Compound 48/80 (*Feldberg & Greengard* 1956); this persistance of histamine also probably reflects the fact that Compound 48/80, as in the rat, is prevented from reaching the endoneurial mast cells via the blood.

Like other mast cells those in the peripheral nerves of the rat also seem to have the capacity to store 5-hydroxytryptamine. Thus recent biochemical studies have demonstrated the presence of this amine in the sciatic nerve of the rat (*Anden & Olsson* 1966) and histochemical studies have shown that mast cells in peripheral nerves give reaction typical for 5-hydroxytryptamine (*Olsson* 1965).

Mast cells of the rat are known to degranulate in response to a variety of stimuli such as mechanical, thermal, bacterial, chemical and irradiation injury (*Uvnäs* 1964). Since such stimuli may also induce peripheral nerve lesions, it seems likely that degranulation of neural mast cells and liberation of their active components can occur in certain pathological conditions of peripheral nerves. In fact degranulation of mast cells has recently been observed at the site of the primary lesion in sectioned sciatic nerves of the rat (*Enerbäck et al.* 1965, *Olsson* 1965).

In other r t ti sucs t h is l n dem nstrated that experimentally in

duced mast cell degranulation is followed by vasodilatation and increased vascular permeability for serum proteins characteristics of the acute inflammatory process (*Benditt et al* 1955 *Rowley & Benditt* 1956) These reactions have been attributed in part to the relase of biogenic amines because they can be suppressed by antihistaminics and by serotonin antagonists (for ref see *Spector & Willoughby* 1963 1964) and because similar vascular reactions can be produced experimentally by the injection of histamine and serotonin (*Majno & Palade* 1961) It is thus possible that the liberated biogenic amines from neural mast cells participate in the development of edema which occurs in some pathological conditions of peripheral nerves

SUMMARY

The morphological changes of mast cells in the peripheral nervous system of the rat induced by a histamine liberator Compound 48 80 were studied after injections by various routes The principal findings were as follows

1 Intraperitoneal injections of Compound 48/80 were followed by degranulation of mast cells in the epineurium and the perineurium of large peripheral nerve trunks and in small peripheral nerve branches in the skin as well as of mast cells in the capsule of dorsal root ganglions On the other hand mast cells in the endoneurium of the nerve fasciculi remained morphologically unchanged even after prolonged administration of Compound 48 80 leading to a depletion of mast cells in the epineurium and the perineurium

2 Microinjection of Compound 48 80 into the sciatic nerve was followed by degranulation of endoneurial mast cells for a long distance along the nerve whereas control injections of Ringer solution only led to slight local changes

3 Application of Compound 48/80 to the surface of exposed sciatic nerves did not result in degranulation of endoneurial mast cells whereas marked morphological changes occurred in mast cells of the epineurium and the perineurium

The present study thus revealed a difference in reaction pattern between mast cells in the endoneurium and mast cells in the external sheaths of peripheral nerves after intraperitoneal injections of Compound 48 80 This different response does not seem to be qualitative Instead it seems most likely that the perineurium and the endoneurial blood vessels have the capacity of denying the histamine liberator access from the blood and from the surrounding tissue fluids to the endoneurial mast cells

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THE EFFECT OF THE HISTAMINE LIBERATOR, COMPOUND 48/80 ON MAST CELLS IN SECTIONED PERIPHERAL NERVES

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Received 22 vi 66

Recent experimental studies have revealed that mast cells participate in the tissue reactions occurring in different pathological conditions of peripheral nerves (*Gamble & Goldby* 1961 *Boschi* 1964, *Enerbäck et al* 1964 1965, *Olsson* 1965 1966 a) Mast cells in rat sciatic nerves react in two different ways to sectioning or crush injury At the site of the trauma there is an early degranulation of both epineurial perineurial and endoneurial mast cells In addition there is a late reaction with a marked increase in the number of endoneurial mast cells throughout the distal part of the nerves (*Enerback et al* 1964, 1965 *Olsson* 1965) Accumulations of endoneurial mast cells have also been observed in a peripheral neuropathy in the rat experimentally induced by isonicotinic acid hydrazid (INH) (*Olsson* 1966 a)

One characteristic feature of mast cells is their fragility and reactivity to a large variety of physical and chemical agents, resulting in degranulation of the cells (*Uvnas* 1964) at the same time the stored biogenic amines of the mast cells are transformed from a bound to an active free form (*Goth* 1964) One of the most potent and in experimental work most widely used of these chemical agents is Compound 48 80 (*Baltzy et al* 1949)

The aim of the present investigation was to characterize the newly formed mast cells in sectioned sciatic nerve of the rat in respect to their reactivity towards the histamine liberator Compound 48 80

MATERIAL AND METHODS

48 Sprague Dawley rats of both sexes were used They weighed between 150 and 2?5 gram at the beginning of the experiments They were fed on commercial rat pellets and water ad libitum The right sciatic nerve of all rats was sectioned high up in the thigh under ether anesthesia 36 rats were treated with the histamine liberator Compound 48 80[1] dissolved in Ringer solution The remaining 12 rats

Compound 48 80 was kindly supplied by The Wellcome Research Laboratories Beckenham Kent England

[1] Compound 48 80 a condensation product between paramethoxyphenethylmethyl amine and formaldehyde

served as controls. The rats were killed at suitable intervals after nerve section and the two sciatic nerves were taken for histological examination. In some of the rats the common peroneal, tibial and sural nerves were studied as well. The following experimental series were employed:

Intraperitoneal injections. Two experimental series received different dosage schedules. In the first series, rats were given daily injections of Compound 48 80 starting on the day of the operation. Groups of rats were treated for 2, 3 and 4 weeks ... injection ...

... ves. Rats were studied ... atic nerve was exposed and about 0 5 ml of a solution containing 100 micrograms Compound 48 80 per ml was flooded around the nerve. The fluid was exchanged every fifth minute. Rats were sacrificed after 5 and 15 minutes and pieces of tissue were taken for examination.

Histological technique. The technique was the same as used in a previous investigation (*Olsson* 1966 b). Briefly, the tissues were fixed in an acetic acid formation mixture and paraffine sections were stained with *toluidine blue*. ...

... in sections from the distal part ... counted in each rat and classified as normal or degranulated. *The criterion* for degranulation was the presence of granules outside the cells. The percentage degranulation of endoneurial mast cells was calculated.

Even in sections from normal tissues one finds a *few mast cells* with a small number of ejected granules. Such extracellular mast cell granules may represent an artifact caused by damage to the cells post mortem (*Michels 1938*). Consequently the proportion and distribution of such cells in the distal endoneurium of sectioned sciatic nerves had first to be determined in rats not treated with *Compound 48 80*.

Degranulated mast cells were observed in all cases most often *located* adjacent to the section surfaces obtained when sampling the material. The vast majority of the cells had only a few ejected granules. The proportion of endoneurial *mast* cells with ejected granules in the whole distal part was low in all rats examined 2 to 12 weeks after nerve section (Table 1).

TABLE 1

Percentage of Endoneurial Mast Cells with Ejected Granules in the Distal Part of Sectioned Sciatic Nerves at Various Times after Operation. Each Group Comprises 3 Control Rats not given Compound 48 80

Time after nerve section (weeks)			
2	4	6	12
2 1	2 5	1 8	[illegible]
2 2	3 1	2 4	[illegible]
3 6	6 6	3 4	[illegible]

RE LTS

Rats given daily injections of Com ... d 48 80 ...
section developed similar genera ... ns as d...
normal rats (*Olsson* 1966 b). In ... eries w...
micrograms Compound 48 80 at ... us time



Figs 1 a and b
Degranulated endoneurial mast cells in the distal part of sectioned sciatic nerves after daily intraperitoneal injection of Compound 48 80 for 2 weeks after operation Toluidine blue

rats died 10, 15 and 30 minutes respectively after the injection The others developed very strong general signs and when killed 5 hours after the injection, they were markedly drowsy and had a profound edema of the skin

Intraperitoneal injections Rats injected daily with Compound 48 80 2 3 and 4 weeks after nerve section showed an almost complete depletion of endoneurial mast cells in the distal part of the sectioned sciatic nerve and of epineurial and perineurial mast cells in both sciatic nerves

In the distal part of the sectioned nerves the vast majority of remaining endoneurial mast cells were degranulated with a large number of granules scattered around the cells (Fig 1) Degranulated endoneurial mast cells were observed throughout the distal part of the sciatic nerves and in the examined proximal parts of their peripheral branches Mast cells without signs of degranulation were extremely rare

In the central part of the sectioned nerves most endoneurial mast cells were of normal appearance However in the vicinity of the lesion some of the cells contained cytoplasmic vacuoles and irregular cytoplasmic borders No mast cells could be identified at the site of the lesion In the epineurium and the perineurium of both nerves scattered metachromatic cytoplasmic inclusions could be detected in a few remaining mast cells

In contrast no signs of degranulation were found for endoneurial mast cells in the non operated sciatic nerve from the same rats

Rats given one injection of 200 micrograms Compound 48 80 at various times after nerve section displayed a heavy degranulation of epineurial mast cells, with scattering of the majority of their granules



3.

Figs 2 3

Fig 2 Numerous heavily degranulated endoneurial mast cells in the distal part sectioned sciatic nerve after one injection of Compound 48 80 4 weeks aft operation Toluidine blue

Fig 3 Numerous mast cells in the distal part of sectioned sciatic nerve after 1 injection of Compound 48 80 12 weeks after operation Some mast cell remain unchanged Toluidine blue

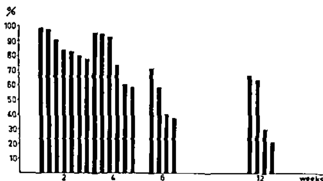


Fig 4

Percentage degranulated endoneurial mast cells in the distal part of sectioned sciatic nerves of rats given one intraperitoneal injection of Compound 48 80 at various times after operation Each column represents one rat

around the cells Not a single intact epineurial mast cells was found in these rats As in the preceding series, endoneurial mast cells of the non operated sciatic nerve appeared entirely normal

Rats injected 2 and 4 weeks after nerve section and killed 5 hours later revealed marked degranulation of endoneurial mast cells in the distal part of the operated nerve (Fig 2) The number of ejected granules seemed to be about the same as for epineurial mast cells Such heavily degranulated endoneurial mast cells were observed throughout the distal part

In the corresponding groups of rats injected 6 and 12 weeks after nerve section, endoneurial mast cells in the distal part were in various stages of degranulation Usually, only a few granules were detected outside the cytoplasm of the cells (Fig 3) In addition several mast cells were identified without signs of degranulation The degree of degranulation did not seem to be so advanced as for epineurial mast cells Most of the degranulated cells seemed to be localized around endoneurial blood vessels

The rats which died 5, 10 and 30 minutes after the injection showed a markedly smaller number of degranulated cells in the distal endoneurium and the cells did not seem to be so heavily degranulated as in the rats killed after 5 hours In contrast there was a marked degranulation of epineurial mast cells

An intense degranulation of mast cells were observed at the site of the primary lesion to the nerves

Quantitative determinations Endoneurial mast cells were counted in the distal part of the sectioned nerves and classified as normal or degranulated The proportion of degranulated cells among mast cells counted consecutively was determined in rats given one intraperitoneal injection of Compound 48 80 at various times after nerve section The result is given in Fig 4

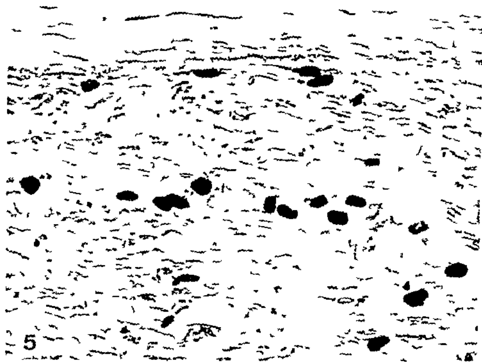


Fig 5

Morphologically unchanged endoneurial mast cells in the distal part of sectioned sciatic nerve 200 weeks after operation Local application of Compound 48/80 around the exposed nerve Toluidine blue

As Fig 4 illustrates the vast majority of endoneurial mast cells are degranulated in the distal endoneurium 2 and 4 weeks after section At 6 and 12 weeks after section the number of degranulated cells is lower

Direct application of Compound 48/80 to the surface of exposed sectioned sciatic nerves Marked degranulation occurred in epineurial and perineurial mast cells of all rats after the direct application of Compound 48 80 for 5 or 15 minutes Endoneurial mast cells in adjacent parts of the distal part on the other hand did not show any morphological deviation from those in control rats not treated with Compound 48 80 (Fig 5) At the site of the primary lesion however mast cells were frequently degranulated in the rats with nerves sectioned 20 weeks earlier

DISCUSSION

Previous investigations of mast cells in sectioned sciatic nerve of the rat have demonstrated a large increase in the number of endoneurial mast cells througout the distal degenerating part (*Enerback et al* 1964 1965) During the first weeks after sectioning these cells are morphologically characterized by their roundness and small size and by the presence of a scanty loose granulation in the cytoplasm In the later

stages the cells are larger and the cytoplasm is crowded with granules These granules show a strong toluidine blue metachromasia that is resistant to alcohol dehydration, and a persistance of dye binding and metachromasia at a low pH These characteristics can be used to differentiate the endoneurial mast cells from other metachromatic structures in sectioned peripheral nerves (*Enerback et al* 1965)

Mast cells in other tissues of the rat are known to be rich in histamine (for ref see *Padawer* 1957, 1963 *Riley* 1959, *Smith* 1963, *Bloom* 1965) Biochemical determinations have revealed a marked increase in the histamine content of the distal part of sectioned sciatic nerve in the rat during the first weeks after operation (*Kwiatkowsky* 1943) The rise in the histamine level seems to run parallel to the increase in the number of endoneurial mast cells which occurs in sectioned nerves This suggests that the newly formed endoneurial mast cells like other mast cells in the rat, contain histamine

Moreover the newly formed endoneurial mast cells in the distal part of sectioned sciatic nerve of the rat seem to contain serotonin (5 hydroxytryptamine, 5-HT) Thus, a good correlation has been reported between the increases in the serotonin level and in the number of endoneurial mast cells at various times after nerve section (*Anden & Olsson* 1966) These cells also give histochemical reactions typical for tryptamines and reaction similar to those of other rat mast cells known to be rich in serotonin (*Olsson* 1965)

In a previous study, disrupted endoneurial mast cells were observed during the first hours after nerve section in the traumatized region (*Enerback et al* 1965) There is only a small proportion of mast cells with ejected granules in the distal endoneurium of such rats 2 to 12 weeks after nerve section the majority appearing in the vicinity of the section surface obtained when sampling the material Thus the present finding of marked degranulation of most endoneurial mast cells throughout the distal part of the sectioned sciatic nerves must be ascribable to the histamine liberator injected by the intraperitoneal route

In contrast endoneurial mast cells in normal peripheral nerves of the rat are resistant to the action of intraperitoneal injections of Compound 48 80 (*Olsson* 1966 b) Since microinjection of Compound 48 80 into the sciatic nerve is followed by a widespread degranulation of endoneurial mast cells it was assumed that the resistance of these cells to intraperitoneal injections of Compound 48.80 is due to the compound being prevented from reaching the endoneurial mast cells from the blood and from the surrounding tissue fluids (*Olsson* 1966 b) The obstruction from the surrounding tissues was thought to be the perineurium This consists to a large extent of closely apposed flattened cells coated with a basal membrane which surround peripheral nerve fasciculi in multiple cellular layers (for ref see *Lehman* 1957, *Shanthaveerappa & Bourne* 1962, 1963, *Thomas* 1963, *Martin* 1964, *Gamble*

1964 *Waggener et al* 1965) In addition it was assumed that the endoneurial blood vessels have the capacity to prevent the histamine liberator from gaining access to the endoneurial mast cells from the blood

The present finding of degranulated endoneurial mast cells in sectioned nerves after intraperitoneal injection of Compound 48/80 indicates that nerve sectioning is accompanied by changes in the permeability of blood vessels leading to a leakage of the histamine liberator into the endoneurium In a parallel fluorescence microscopic study the permeability of blood vessels in normal and sectioned sciatic nerves of the rat was outlined by direct observation of the distribution of intravenously injected labelled serum albumin (*Olsson* 1966c) In normal nerves fluorescent albumin was confined to the lumen of endoneurial blood vessels but in the epineurium it was also found in connective tissue structures outside the blood vessels Immediately after nerve section there was a very marked outpouring of labelled albumin at the site of lesion to the nerves and a rapid spread of the fluorescent albumin in the distal endeneurium Small amounts of fluorescent albumin was also observed around endoneurial blood vessels in the distal endoneurium 2 to 12 weeks after nerve section This indicated an increased permeability of endoneurial blood vessels in the distal degenerating part of the nerves Thus, in the present experiments it seems likely that Compound 48/80 after intraperitoneal injection leaked into the endoneurium either from blood vessels at the site of the primary lesion to the nerve or from leaking blood vessels in the distal part

Since the perineurium also is heavily damaged at the site of the lesion of sectioned nerves, the degranulation of endoneurial mast cells in the distal part could be due to leakage of Compound 48/80 from tissue fluids surrounding the peripheral nerves However local flooding of Compound 48/80 around the sectioned nerves only resulted in a local degranulation of endoneurial mast cells at the site of the section no changes were detected in endoneurial mast cells in the distal part This indicates that the perineurium in the distal part of sectioned nerves preserved its capacity as a diffusion barrier to Compound 48/80 Detailed morphological end enzyme-histochemical investigation of sectioned rat sciatic nerves has also failed to reveal any changes of the perineurial epithelium in the distal part (*Shanthaveerappa & Bourne 1964*)

It has been shown experimentally that the mast cell degranulation induced in other rat tissues by Compound 48/80 is a complex mechanism dependant on energy requiring processes (for ref see *Uvnas* 1961 *Westerholm* 1964) The present finding of degranulation after injection of Compound 48/80 thus suggests that the newly formed endoneurial mast cells in sectioned nerves are equipped with the enzyme systems involved in the degranulation mechanism

The functional significance of the greatly increased number of endoneurial mast cells in sectioned nerves is still far from clear As discussed earlier it is possible that liberated products from these cells

influence some of the changes occurring in the tissues of the distal part of the sectioned nerve (*Enerbäck et al* 1965 *Olsson* 1965) Although it has not yet been demonstrated that substances are continuously liberated from the mast cells the present study clearly shows that degranulation of the endoneurial mast cells can be elicited by stimulation with a histamine liberator Accordingly there must also be a liberation of biogenic amines from these cells after the injection of Compound 48 80 This affords a possibility for further studies of the significance of the increased number of endoneurial mast cells in states of enhanced liberation of stored active products

SUMMARY

A study was made of the effect of the histamine liberator Compound 48.80 on the numerous mast cells which appear in the endoneurium of the distal part in sectioned rat sciatic nerves The principal findings were as follows

1 Daily interperitoneal injections of Compound 48 80 for up to 4 weeks after nerve section were followed by an almost complete depletion of mast cells in the distal part of the sectioned nerves and of epineurial and perineurial mast cells in both nerves Endoneurial mast cells in the control nerves from these rats did not show any morphological alterations

2 Injectione of a single large intraperitoneal dose of Compound 48.80 into rats at 2 4 6 and 12 weeks after nerve section was followed by degranulation of endoneurial mast cells in the distal part of the sectioned nerves and of epineurial mast cells in both nerves Endoneurial mast cells in the control nerves remained entirely unchanged

3 Local application of Compound 48 80 to the surface of exposed sectioned sciatic nerves was followed by degranulation of epineurial and perineurial mast cells The underlying endoneurial mast cells in the distal part remained unaffected

Thus the newly formed endoneurial mast cells in the distal part of sectioned sciatic nerves like most other mast cells in the rat have the capacity to degranulate as a response to the histamine liberator Compound 48 80 The degranulation of endoneurial mast cells in sectioned nerves after intraperitoneal injection of Compound 48.80 most probably reflects an increased permeability of endoneurial blood vessels in the sectioned nerves leading to leakage of the histamine liberator into the distal part

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IMMUNIZATION OF GUINEA PIGS WITH FOOT AND MOUTH DISEASE VIRUS SUBJECTED TO PHOTODYNAMIC INACTIVATION

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Received 11 vii 66

A number of reports have been published in recent years concerning photodynamic inactivation of viruses (4 5 7 9 10 12 13) *Hiatt et al* (3) classified some animal viruses according to the rate of inactivation by constant irradiation and dye concentration (toluidine blue) and gave a brief survey of previously published works in the field of photodynamic inactivation *Wallis & Melnick* (14) examined the photosensitivity of enteroviruses in connection with the dyes proflavin and toluidine blue and demonstrated an increased photosensitivity of polio virus when the virus suspensions had passed an anion exchanger before the addition of dye (toluidine blue) and the application of light (12)

Apparently only few works have been published concerning the immunizing capacity of virus subjected to photodynamic inactivation *Perdrau & Todd* (8) found good antigenic response in ferrets and dogs vaccinated with canine distemper virus inactivated by methylene blue and light In 1937 *Galloway* (2) described experiments in which foot and mouth disease virus (FMDV) was inactivated photodynamically by means of a proflavin (3 7 diamino 10 cyano 5 methylacridin) This virus was tested for its immunizing effect on guinea pigs but found to be less effective than after inactivation by formalin In 1963 *Wallis et al* (11) published the results of a study on the antigenic effect on mice of an influenza vaccin produced from virus inactivated by toluidine blue

The aim of the present study was to establish whether it might be possible to obtain an inactivated FDMV with a good immunizing effect when inactivation was performed by means of photodynamic action with proflavin and a suitable combination of some of the factors of significance for the photodynamic effect proflavin source of light and irradiation distance

MATERIAL AND METHODS

Virus The virus used throughout the experiments was type C FDMV (coming from the Institute's routine production of vaccine antigen) multiplied in cultures of bovine tongue epithelium according to *Frenkel's* (1

Titration of virus and inactivation control Both for determination of the infectious titre of the virus suspensions and for determination of the inactivation of the virus suspensions used for vaccination use was made of calf kidney tissue cultures in Earle's salt solution to which was added 0.5 per cent lactalbumin hydrolysate 0.01 per cent yeast extract 1 per cent inactivated horse serum and antibiotics The infectious titre was determined by inoculating 0.1 ml on each of four tissue cultures per tenfold dilution of the virus suspension and was expressed as $\log_{10}$ TCD_{50}/0.1 ml according to *Kärber's* method (6) For inactivation control 5-10 tissue cultures were each inoculated with 0.2 ml of virus suspension which had been subjected to photodynamic inactivation After incubation for 48 hours at 37° C the cultures were inspected for cytopathic changes

Vaccination and immunity The vaccination of guinea pigs with the inactivated virus suspensions was performed subcutaneously with doses of 4.0 1.0 and 0.25 ml Ten guinea pigs were vaccinated with each dose and immunity testing was carried out 16-18 days after vaccination using cattle virus adapted to guinea pigs 10 non-vaccinated animals were inoculated simultaneously as virus controls The virus was injected intracutaneously in one planta When only the inoculated planta was affected complete protection was considered to have been achieved

Irradiation The source of light was a fluorescent lamp (Philips CLD 15W/32) or an ultraviolet lamp (Philips TUW 15/W) In all cases irradiation was carried out after the virus proflavin mixture had been allowed to stand for 24 hours at 4° C The temperature of the medium during irradiation was 24-27° C

Buffer (pH 8.3) 100 ml of 0.2 M Tris (hydroxymethyl) aminomethane 80 ml of 0.1 M HCl and water to 1000 ml

Albumin solution 4 per cent aqueous solution of bovine albumin diluted with buffer to the desired concentrations

Dye Proflavin (3.6 diaminoacridine) from which was prepared a 0.01 per cent stock solution in buffer this solution was stored in the dark at 4° C

EXPERIMENTS AND RESULTS

Inactivation Experiments

In a preliminary experiment it was investigated whether proflavin without the presence of light affected the infectious titre of the virus Table 1 shows that under the chosen conditions no inactivation could be demonstrated

Table 2 shows that irradiation with fluorescent lamp without the presence of proflavin caused no significant change in the infectious titre during the periods selected In these experiments 10 ml of virus suspension was placed in an open petri dish (diameter 9 cm) which was rotated slowly under the source of light at a distance of 8 cm

TABLE 1

Effect of Proflavin on the Infectious Titre of FMDV in the Absence of Light

Storage time in days at 4° C	0	1	2	3	4	5
	$\log_{10}$ TCD_{50}/0.1 ml					
VB 1 µgP/ml	6.5	6.5	6.5	6.8	6.8	6.0
VB 2 µgP/ml	7.0	6.5	6.3	6.5	7.0	6.8
VB	6.5	6.8	6.3	6.5	6.8	6.8

VB = virus suspension + buffer 1 + 1

P = proflavin

TABLE 2

Results from Irradiation of FMDV with Light from a Fluorescent Lamp and without the Presence of Proflavin

Irradiation time in minutes	0	5	10	15	20	25	30
	$\log_{10}$ TCD$_{50}$/0 1 ml						
VB	6 5	6 3	6 0	6 3	6 0	6 0	6 0
VA	6.3	6 0	6 0	5 8	6 0	6 0	6 0

VA = virus suspension + 1 per cent bovine albumin solution 1 + 1
VB = virus suspension + buffer 1 + 1

TABLE 3

Course of Inactivation of FMDV with Proflavin Irradiated with Fluorescent Lamp

	$\log_{10}$ TCD$_{50}$/0 1 ml			
Irradiation time in minutes	VB 1 µgP/ml	VB 2 µgP/ml	VA 1 µgP/ml	VA 2 µgP/ml
0	5 5	5.3	5 5	5 8
5	2 8	3 3	3 3	3 3
10	1 5	0 8	1 5	1.3
15	< 0 5		0 5	
20		—		

VB = virus suspension + buffer 1 + 1
VA = virus suspension + 1 per cent bovine albumin solution 1 + 1
P = proflavin — no cytopathogenic degeneration

TABLE 4

Course of Inactivation of FMDV with and without Proflavin Irradiated with Ultraviolet Light

	$\log_{10}$ TCD$_{50}$ 0 1 ml			
Irradiation time in minutes	VB	VA	VA 1 µgP ml	VA 2 µgP/ml
0	5 5	6 0	6 0	6 0
2	2 5	2 8	2 0	2.3
4	1 0	2 0	0 8	< 0 5
6	0 8	1 0		
8		0 3		
10				

V = virus suspension buffer 1 + 1
VA = virus suspension 1 per cent bovine albumin solution 1 + 1
P proflavin — no cytopathogenic degeneration

Tables 3 and 4 give the results of irradiation by fluorescent lamp of virus in the presence of proflavin under the same experimental conditions as above. It will be seen from the tables that the varying proflavin concentrations of 1 and 2 µg/ml caused only insignificant, small differences in the inactivating effect Table 3 also shows comlete inactivation of virus in 20 minutes or less by irradiation with fluorescent lamp, and Table 4 shows that the rate of inactivation during irradiation with ultraviolet light alone was not significantly different from that caused by ultraviolet light plus proflavin

Bovine albumin added to protect the antigenic capacity of the virus during the irradiation, seemed to have little or no influence on the course of inactivation

TABLE 5

Course of Inactivation of FMDV with Proflavin Irradiated during Passage through Glass Spiral

Passage time in minutes for 50 ml	0	13	11 5	9 5	8 5	7 5
V B 1 µgP/ml	6 3*	—	—	—	+	+
V B 2 µgP/ml	5 8*	—	—	—	—	+

V B = virus suspension + buffer 1 + 1
P = proflavin
+ = not inactivated, — = inactivated
* = $\log_{10}TCD_{50}$/0 1 ml

In order to ascertain whether a change in the irradiation conditions would give a better immunizing antigen, inactivation was performed by irradiation of the virus suspension during its passage through a spiral of ordinary glass tubing with a wall thickness of 1 mm and a bore of 3 mm The source of light was the above-mentioned fluorescent lamp placed in the centre of the spiral, 1 cm from the convolutions Just before the virus suspension entered the spiral, air bubbles were introduced by means of a T-tube These distributed the flowing fluid into segments thus preventing the lamina flow in the spiral from being to rapid and ensuring a uniform irradiation As will be seen from Table 6 the use of the spiral did not result in an antigen with a better immunizing effect than that achieved by using the petri dish method Table 5 shows that a passage time of 9 5 and 8 5 minutes for 50 ml of virus suspension with 1 and 2 µg proflavin per ml, respectively results in complete inactivation of the infecting ability of the virus Since the capacity of the spiral was 20 ml the irradiation time (i e the time taken by an air bubble to pass through the spiral) resulting in complete inactivation was from 3 4 to 3 8 minutes

TABLE 6

Immunization Results Achieved by Vaccination of Guinea Pigs with LMDV Subjected to Photodynamic Inactivation

( ) 2 gives the i f ti us titre ($log_{10}TCD_{50}$/0 1 ml) for the original LMDV suspension, and in Cols 3 and 4 the components of the vaccine are indicated.

Vaccine No	Virus titre	Composition of vaccine Virus	Composition of vaccine μgP/ml	Inactivation method	Dose of vaccine ml	% immune guinea pigs Vaccinated	% immune guinea pigs Controls
1	6 8	V A	2 5	180 min in daylight	4 1 ¼	0 0 0	0
2	7 0	V A	2 5	60 min in daylight	4 1 ¼	100 90 60	0
3	7 0	V M	2 5	60 min in daylight	4 1 ¼	100 80 40	0
4	6 5	V A	2 0	45 min under fluorescent lamp	4 1 ¼	100 80 60	0
5	6 5	V A	1 0	45 min under fluorescent lamp	4 1 ¼	100 70 50	0
6	6 8	V A	2 0	10 min under ultraviolet lamp	4 1 ¼	20 10 0	0
7	6 8	V A	1 0	Glass spiral and fluorescent lamp	4 1 ¼	100 70 60	0

V A — virus suspension + 1 per cent bovine albumin solution 1 + 1
V M — virus suspension + Baker's medium 1 + 1
P — proflavin

Immunizing Experiments

The results given in Table 6 shows the immunizing effect obtained by vaccination of guinea pigs with virus subjected to photodynamic inactivation In every case the inactivation process was complete, juged in relation to cytopathogenic effect in tissue culture In the first three experiments the virus was inactivated by means of daylight In experiments Nos 4, 5, and 6, the virus suspensions (70–75 ml) were placed during inactivation in petri dishes (diameter 14 cm) at a distance of 8 cm under the source of light mentioned in the table The size of the petri dishes in relation to the volume of the fluid in these experiments gives a greater thickness of fluid than that described for the corresponding experiments stated in the Tables 2 and 3 This deviation was made only to give a volume of virus suspension great enough for vaccination of the guinea pigs In experiment 7 the virus was inactivated by irradiation during passage through the above-mentioned spiral The source of light was the previously mentioned fluorescent lamp and the passage time for 50 ml was about 12 minutes

It will also be seen from Table 6 that, under the conditions of this study, treatment with ultraviolet light yields an inactivated antigen with poor immunizing effect on guinea pigs (vaccine No 6) In this study there would appear to be no differences in the immunizing effect of the inactivated virus suspensions which can be attributed to the different proflavin concentrations (Nos 4 and 5), the auxiliary substance bovine albumin (Nos 2 and 3), or to the inactivation methods (Nos 3, 5, and 7) except for the destruction of immunogenicity by ultraviolet light (No 6) However, the immunizing effect is destroyed if the virus is irradiated in daylight for a longer period than that found adequate (No 1)

The vaccination experiments on guinea pigs with FMDV inactivated by means of photodynamic action, which form the basis of this report, are not limited to those shown in Table 6 A further 35 experiments have been performed, all of which show immunizing results corresponding to those shown for vaccines Nos 2, 3 4, 5, and 7 in Table 6

The present study confirms *Galloway's* (2) demonstration of an antigenic effect on guinea pigs of FMDV subjected to photodynamic inactivation The author has demonstrated that the immunizing effect of the antigen used was satisfactory, since a dose of 0 25 ml applied subcutaneously provided protection to about 50 per cent of the guinea pigs

To the authors knowledge no studies have been carried out with FMDV suspensions from the point of view of the possible occurrence of dye-absorbing components which might influence the photodynamic effect An investigation of polio virus suspensions made by *Wallis & Melnick* (13) showed that toluidine blue may be both photodynamically active against that virus, depending on the content in the medium

of dye absorbing components, which can be removed by treatment with an anion exchanger Similar studies with FMDV may result in the finding that further dyes are photodynamically active against that virus

SUMMARY

Suspensions of foot and mouth disease virus to which proflavin had been added were irradiated with visible light until virus had lost its infecting capacity in calf kidney tissue cultures Vaccination of guinea pigs with these suspensions resulted in good immunity whereas application of ultraviolet light caused the virus to lose not only its infectivity but its immunizing effect as well

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EXPERIMENTAL TOXOPLASMOSIS

Effect of Inoculation of Toxoplasma in Seropositive Rabbits

By

GUNNEL HULDT

Received 20 vi 66

Experimental toxoplasmosis in rabbits seronegative (DT[1] CFT[2]) before inoculation was studied in a previous investigation (3) It was found that the early multiplication and spread of *Toxoplasma* primarily occurs in the regional lymph node and that the parasite is transported in lymph and blood mainly intracellularly within lymphocytes It was further demonstrated that Toxoplasma causes considerable damage to reticulo endothelial and lymphatic tissues which showed both necroses and severe reaction appearing mainly as proliferation of phagocyting cells (macrophages monocytes) and decrease of mature lymphocytes with signs of accelerated lymphocyte production

It seems logical to proceed with a corresponding investigation on seropositive animals A study of the host parasite interaction in reticulo endothelial and lymphatic tissues of such animals might be a way of elucidating some features of the complex defence mechanism in toxoplasmosis

When planning this study certain facts known about immunity in toxoplasmosis had to be considered In the first place it is well known that animals which have experienced toxoplasmic infection show a considerable degree of immunity to challenge However it has been demonstrated that injections of large infective doses of parasites or strains of high virulence can break through the defence (2) It is also known that immunization of animals with killed *Toxoplasma* organisms gives a good antibody response but often incomplete or no resistance to inoculation with living Toxoplasma This has been shown by *Levaditi et al* 1928) *Cutchings & Warren* (1956) and *Waldfuhr* (1957) For the present investigation it was therefore decided to use animals that were seropositive after past infection as well as after immunization with killed Toxoplasma

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1 DT = dye test
2 CFT = complement fixation test

MATERIAL

A total of 23 rabbits were used for the experiments The animals were 6–12 months of age weighing about 2 5 kg As in the previous investigation (3) they were obtained from a few well known breeders and during the period of study were kept isolated and fed on turnips oats and hay

Sabin s *Toxoplasma* strain RH was used throughout

METHODS

The methods used have been described in a previous investigation (3) However instead of counting 15 oil immersion fields when making differential cell counts of imprints 1000 cells were counted

EXPERIMENTAL AND RESULTS

Clinical signs of illness, parasite multiplication and tissue reactions were compared in four groups of experimentally infected rabbits, namely

I Seronegative rabbits killed on one of days 6–7 after inoculation, described in an earlier study (3) (Nineteen animals)

II Rabbits with high antibody titres after immunization with killed *Toxoplasma* organisms The animals were immunized as follows Fresh toxoplasmic mouse peritoneal exudate was diluted with saline to con tain about 100 000 parasites per ml To 10 ml of this suspension, 0 125 ml 40 per cent formol solution was added After thorough mixing the suspension was kept in a refrigerator for six hours and 0 4 ml then injected intravenously The injections were repeated once a week until high antibody titres were attained, *i e* DT $\geqq$ 1/1250, CFT $\geqq$ 1/120 These titre levels were reached in 4–6 weeks when challenge inocul ation was performed (Six animals)

III Rabbits with high antibody titres (DT $\geqq$ 1/1250 CFT $\geqq$ 1/120) after natural infection (Eleven animals)

IV Rabbits with low or moderate antibody titres (DT 1/50–1 250 CFT $\leqq$ 1 60) after natural infection (Six animals)

After intracutaneous inoculation with about 500 000 *Toxoplasma* organisms in the foot pad the rabbits were allowed to live for 6 9 days whereafter they were killed by the intravenous injection of 3 5 ml mebumal (sodium salt)

Table 1 shows the investigations carried out on the animals of groups II IV For various reasons certain animals were not subjected to all investigations as a rule because material could not be obtained This is the case with superior iliac lymph node and lymph As for temperature controls and counting of white blood cells in certain other animals these examinations were not considered necessary at the actual time

Clinical course The symptomatology of experimental toxoplasmosis in seronegative rabbits group I) has already been described (3) After challenge all animal of groups II and IV (except rabbit 161) became

seriously ill They reacted in the same way as seronegative animals All rabbits had running noses and signs of conjunctivitis from day 4, one animal of group IV diarrhoea as well Temperature controls carried out on the rabbits of group II showed a rise of body temperature to 40° C from day 2 or 3, lasting throughout the trial period Due to their poor clinical condition, two animals of group IV had to be killed on day 6 One animal of group II died spontaneously on day 6, and one of group IV on day 7

TABLE 1
Examinations Performed on Animals in Experimental Groups II–IV

Experimental group	Number of animals	Temp controls	Hematological investigation		Imprints		Isolations	
			Blood	Lymph	Popl l n	Iliac l n	Blood	Lymph
II	6	6	6	4	6	6	6	4
III	11	5	5	8	11	8	11	10
IV	6	1	6	5	6	5	6	5

Animals of group III and rabbit 161 of group IV seemed to be in good condition during the whole experiment Daily temperature controls in five animals did not reveal any reactions (fluctuations in no case more than 0 6° C)

Lymph nodes Enlargement of the popliteal lymph node was demonstrated in all animals This was most pronounced in groups I, II and IV (except 161), in which the organ was enlarged to 3–4 times the normal size These nodes were oedematous, and in groups II and IV (except 161) necroses were more numerous and larger than in the seronegative rabbits (group I) —In group III and rabbit 161, the popliteal lymph nodes, oedematous but without necroses, were enlarged to about double the normal size

The superior iliac lymph node was *enlarged to about double the* normal size in all animals of groups II and IV (except 161) and in 8 rabbits of group III Numerous necroses were seen in nodes from groups II and IV but in not nodes from group III

Fig 1 shows means and standard errors of haemocytoblast, large lymphocyte and plasma cell ratios in imprints from the regional lymph nodes both in seronegative animals (group I) and in groups II–IV The area covered by mean and standard error for a normal material (reported in a previous paper 3) is indicated in each diagram As can be seen, haemocytoblasts were found in proportions significantly greater than for normal animals However, the significance was of a high degree ($P < 0.001$) in groups I and III only The large-lymphocyte ratio was also increased for all groups, a high degree of significance being demonstrated only for groups I and IV As to plasma cells, a highly significant increase was demonstrated in groups II and III

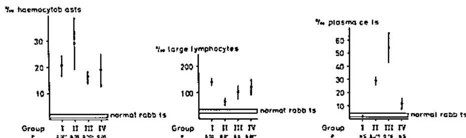


Fig 1

Means and standard errors of haemocytoblast, large lymphocyte and plasma cell ratios in imprints from popliteal lymph nodes of the four experimental groups The shaded area covers the mean and standard error of a normal material

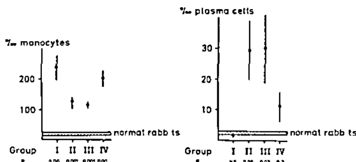


Fig 2

Means and standard errors of monocyte and plasma cell ratios in spleen imprints from the four experimental groups The shaded area covers the mean and standard error of a normal material

Toxoplasma was seen abundantly in popliteal and superior iliac lymph nodes from groups II and IV (except 161) The parasites were demonstrated both extra- and intracellularly throughout the organ In corresponding lymph nodes from rabbit 161, *Toxoplasma* was not seen In 10 of 11 imprints from popliteal lymph nodes and in 6 of 8 preparations from superior iliac lymph nodes from group III *Toxoplasma* was seen though the occurrence was sparse and limited to the periphery near the marginal sinus

Spleen With the exception of rabbit 161 the spleens from all animals of groups I, II and IV were enlarged to about double the normal size The consistency was softened and the organs showed numerous necrotic foci, which were larger and more numerous in groups II and IV The spleens from rabbit 161 and from the rabbits of group III did not show any macroscopical changes

Fig 2 shows the means and standard errors of monocyte and plasma-cell ratios for groups I IV —The ratio of monocytes was significantly increased for all groups Compared to normal animals, means of plasma-

seriously ill They reacted in the same way as seronegative animals All rabbits had running noses and signs of conjunctivitis from day 4, one animal of group IV diarrhoea as well Temperature controls carried out on the rabbits of group II showed a rise of body temperature to 40° C from day 2 or 3, lasting throughout the trial period Due to their poor clinical condition, two animals of group IV had to be killed on day 6 One animal of group II died spontaneously on day 6, and one of group IV on day 7

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			Blood	Lymph	*Popl l n*	*Iliac l n*	Blood	Lymph
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IV	6	1	6	5	6	5	6	5

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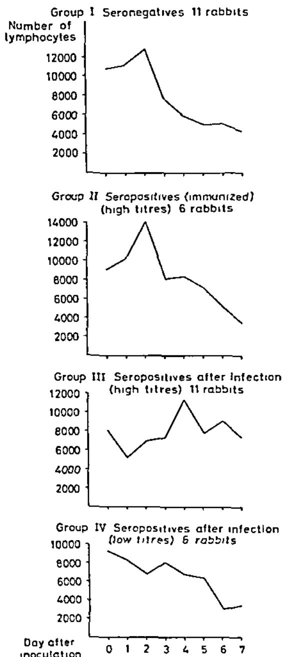


Fig 4

Means of lymphocyte numbers in the peripheral blood on the various days of the trial period in the four experimental groups

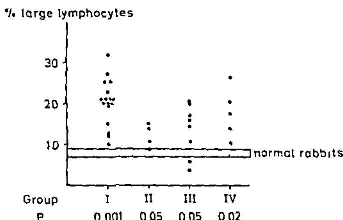


Fig 5

Percentage of large lymphocytes in thoracic duct lymph in the four experimental groups The shaded area covers the mean and standard error of a normal material

preparations peripherally near the capsule and in imprints from two rabbits of group II and two of group IV in the red pulp as well No toxoplasms were found in imprints from rabbit 161 and all rabbits of group III

White blood cell picture It was demonstrated in an earlier investigation (3) that three main types of change in white blood cells appear in experimentally infected toxoplasmic rabbits, namely monocytosis lymphopenia and the appearance of basophilic 'toxic' granules in the pseudo eosinophilic leucocytes A comparison between group I IV shows the following

Monocytes Fig 3 shows the mean numbers of monocytes for each day in each of the four groups The diagrams indicate that beginning on day 4 the rabbits of all groups developed considerable monocytosis The blood monocyte level showed a continuous increase culminating on day 6 in groups II IV In these groups a slight though not significant decrease was observed on day 7 A comparison between mean numbers for days 0 1 and days 6 7 shows highly significant differences ($P < 0.001$) from groups I II and IV For group III, the significance was lower P 0 01 The dotted lines in the diagrams for groups III and IV demonstrate monocyte levels for rabbits 161 and 144 respectively These animals seemed to have the highest degree of resistance and they also exhibited a transient monocytosis on days 5 6

Lymphocytes It will be seen from Fig 4 that groups I, II and IV but not group II reacted with a significant ($P < 0.001$) decrease in lymphocytes during the experiment

Basophilic toxic granules were demonstrated in 70 95 per cent of the pseudo eosinophilic leucocytes in preparations from days 6 7 in

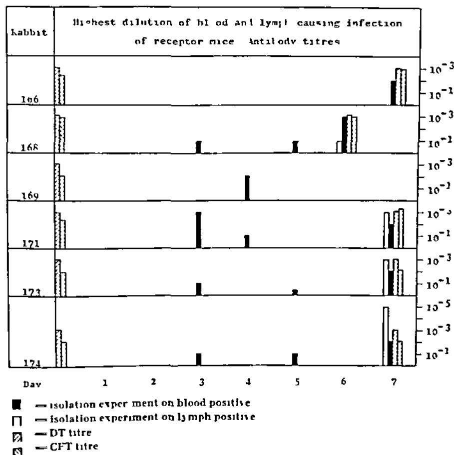


Fig 6

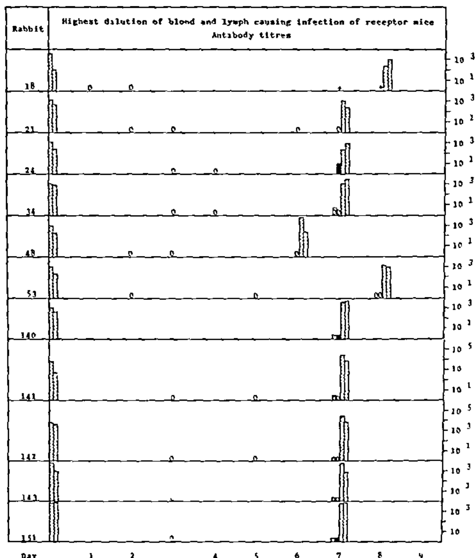
Isolation experiments and antibody titres in the animals of Group II

groups I, II and IV. The corresponding ratio for group III was 10.20 per cent.

Lymph. Fig. 5 indicates that large lymphocytes were found in higher proportions than in normal animals in all four groups. However statistical analysis demonstrated a high degree significance in group I alone. The low degree significance for the remaining groups may have been due to the small number of samples.

Isolations. The results of isolation experiments in group I have been described in a previous paper (3). They do not differ significantly from those obtained from groups II and IV (except 161) of the present investigation.

Fig. 6 shows that all animals of group II developed considerable parasitaemia demonstrated at the earliest on day 3. Toxoplasma was



O — isolation experiment on blood negative
■ — isolation experiment on blood positive
— isolation experiment on lymph negative
— isolation experiment on lymph positive
— DT titre
— CFT titre
+ — isolation experiment blood positive titrations not performed

Fig 7

Isolation experiments and antibody titres in the animals of Group III

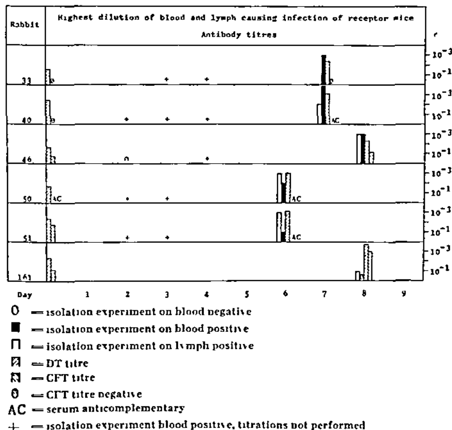


Fig 8

Isolation experiments and antibody titres in the animals of Group IV

also isolated in high titre from lymph in three animals, and in moderate titre in one

Fig 7 presents the results of isolations in group III It will be seen that *Toxoplasma* was isolated from the blood of five animals, but in four of them only late in the experiment (day 6 or 7), and to the extent that titrations were carried out, it seems hat parasitaemia was of a low degree

In only one rabbit (140) was *Toxoplasma* isolated from lymph In five of the six rabbits in which *Toxoplasma* was not isolated from blood or lymph, it was seen in imprints from lymph nodes, indicating parasite multiplication In one rabbit (141) *Toxoplasma* was neither isolated nor seen in imprints

In all animals of group IV, except 161, *Toxoplasma* was isolated from the blood, in three of them from day 2 on, and in the remaining two from day 3 on An attempt at estimating the greatest dilution of

blood from which *Toxoplasma* could be isolated was only performed on the last day of the experiment Fig 8 shows that four animals had heavy parasitaemia on that day On the same day massive infection of the lymph was also demonstrated in five animals Isolation experiments on blood from rabbit 161 were negative, although *Toxoplasma* was isolated from its lymph in dilution 1 10

Antibody titres As to isolations, the antibody titres for group I have been reported on in a previous study (3)

Fig 6 shows that DT titres as a rule remained unchanged during the experiments in group II A rise in CF titres of two or more steps was demonstrated in three rabbits

In group III (Fig 7) a rise in DT titres of two or more steps was seen in two rabbits, and a corresponding rise in CF titres in five rabbits In one animal, however, a decrease in the DT of two titre steps was observed There is no ready explanation for this finding

From Fig 8 it will be seen that the DT titres in group IV increased during the trial period Increase in CF titres, however, could only be demonstrated in three animals Sera were anticomplementary in one rabbit, and in the remaining animals the CFT was still negative on the last day of the experiment —The steepest increase in antibody titre was found in rabbit 161

The main findings can be summarized as follows

1 Over and above the animals of group I (3), severe clinical illness was also found in all rabbits of groups II and IV (except 161)

Rabbit 161 and the animals of group III did not show any clinical signs of illness

2 By means of isolations and imprint technique, abundant parasite multiplication was demonstrated in groups I (3), II and IV (except 161) In group III, parasite multiplication was demonstrated in 10 of 12 animals examined Here, however, it was very sparse, compared to the two other groups

3 Necroses in lymph nodes and spleen were larger and more numerous in groups III and IV than in experimentally infected seronegative rabbits (group I)

4 Considerable proliferation of monocytes both in the spleen and the peripheral blood was demonstrated in all experimental groups

5 Decrease in the blood lymphocyte level was demonstrated in groups I, III and IV A significant increase in large lymphocytes in imprints from lymph nodes and in lymph was demonstrated in all groups, however

6 A significant increase in plasma cells in imprints from lymph nodes and spleen was demonstrated in groups II and III

The results will be discussed in the following paper

SUMMARY

Resistance to challenge inoculation of *Toxoplasma* was studied in three groups of seropositive rabbits. For comparison experimentally infected seronegative rabbits, examined on days 6–7 after inoculation and described in a previous report, are referred to as experimental group I.

The three remaining experimental groups (seropositive rabbits) are:

Group II. Animals with high antibody titres (DT ≧ 1/1250, CFT ≧ 1/120) after immunization with killed toxoplasms.

Group III. Animals with high post-infection antibody titres (DT ≧ 1/1250, CFT ≧ 1/120).

Group IV. Animals with low or moderate post-infection titres (DT 1/50–1/250, CFT ≦ 1/60).

Presence and degree of resistance were determined mainly by studying clinical signs of illness, degree of parasitaemia and multiplication of parasites in lymphatic tissues.

It was found that all animals but one belonging to groups II and IV did not show any signs of protective immunity to *Toxoplasma* infection. The clinical picture, parasitaemia and multiplication of parasites in lymphatic tissues were as severe as in the experimentally infected seronegative rabbits that were reported on in an earlier paper.

All the animals of group III and one of group IV revealed good resistance to challenge inoculation. Clinical signs of illness were not demonstrated during the trial period; parasitaemia, when present, was only of low degree and very few parasites were found in lymphatic tissue.

Cellular reactions were also found in animals of group III with highly modified toxoplasmosis. Thus an increase of monocytes in the peripheral blood and in spleen imprints was seen, together with a relative increase of immature lymphocytes and haemocytoblasts in lymph nodes.

In rabbits with high antibody levels before challenge (groups II and III) an increase of plasma cells as compared to normal uninfected rabbits was found in lymph nodes and spleen.

Toxic granules in pseudo-eosinophilic leukocytes were present in considerable numbers only in animals with severe infection.

Necroses in lymphatic tissues (lymph nodes, spleen) were more numerous and larger in animals with pre-inoculation antibodies than in seronegative rabbits.

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EXPERIMENTAL TOXOPLASMOSIS

Effect of Cortico-Steroids on Rabbits with Varying Degree of Immunity

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It is a well known fact that infections often take a more violent course in animals which have been pre-treated with cortico-steroids. This has been demonstrated for bacterial, viral and protozoan infections. The action of cortico-steroids seems to be complex including both influence on unspecific activities like phagocytosis and involvement of immunological mechanisms. Reduced formation of antibodies has been demonstrated in cortico-steroid treated rodents (2). It is also well known that cortico-steroids have a depressive action on delayed type hypersensitivity presumably by reducing the number of lymphocytes. However, there are still many unsolved problems concerning the effect of these drugs.

The action of cortico-steroids on *Toxoplasma* infection has been studied by a number of investigators. *Kaufman* (1960) found that in experimental ocular toxoplasmosis in rabbits, caused by a strain of low virulence, the local reactions became less pronounced in animals treated with cortico-steroids, but that these animals died in a generalized infection. *Frenkel* (1956) showed that hamsters with chronic infections having resisted challenge inoculation succumbed after prolonged administration of cortisone acetate. The animals died from toxoplasmic pneumonia and encephalitis accompanied by necrosis with deficient inflammatory response. *Metzger et al.* (1963) demonstrated that parasitaemia was more frequent in cortico-steroid treated white rats than in untreated controls. Further, pre-treatment with cortico-steroids has been reported to give better results in isolation experiments on mice (*Eichenwald* 1956, *Frichsen & Harboe* 1953).

As cortico-steroids are known to affect not only antibody production but also cellular defence, it was considered reasonable to study more closely the response of cortico-steroid treated rabbits to *Toxoplasma* infection. In a previous investigation it was shown that there is a fundamental difference regarding the protection against challenge between rabbits with high post-infection titres and animals with the same antibody level after immunization with killed *Toxoplasma* organisms. Seropositive rabbits representing these two groups and seronegative control animals were therefore studied.

MATERIALS

Fourteen rabbits (weight about 1 5 kg) were used for the experiments They came from the same breeders and were kept in the same way as described earlier

Sabin s strain RH was used throughout both for providing toxoplasmin and anti gen for the dye and complement fixation tests The antigen and control antigen for the toxoplasmin tests were the same as those used for the CFT[2] (made from chorio allantoic membranes) sterile filtered through a glass filter size G 5

METHODS

Administration of cortico steroids Cortone (MSD) containing 25 mg cortisone acetate per ml was used Daily injections of 0 5 ml of this preparation per kg body weight were given to all animals beginning 4 days before inoculation and continuing during the whole trial period In order to avoid interfering bacterial infections 50 000 IE penicillin and 6 3 mg streptomycin were injected daily together with the cortone

Toxoplasmin test 0 1 ml of each toxoplasmin and control antigen were injected intracutaneously on the shaved flanks of the rabbits Assessments were made after 48 and 72 hours Classified as positive were reactions consisting of erythema diameter $\geq 10 \times 10$ mm and induration provided that the site of the injected control antigen did not show any reaction

In other respects the same methods as those described in previous papers (12 13 14) were employed

Three experimental groups were investigated

1 Seronegative (DT[1] CFT[2]) rabbits (Five rabbits)
2 Rabbits positive in high titres (DT $\geq$ 1/1250 CFT $\geq$ 1/120) after immunization with killed toxoplasma organisms (Four rabbits)
3 Rabbits positive in high titres (DT $\geq$ 1/1250 CFT $\geq$ 1/120) after previously sustained natural infection (Five rabbits)

All animals were bled for DT and CFT before the toxoplasmin tests The cortisone treatment was begun 1 5 days after the assessment of this test This day is indicated as day —4 On day 0 the rabbits were inoculated with *Toxoplasma*—Beginning on day —4 samples of blood were taken each day from an auricular vein for white blood cell examination Daily measurements of the rectal temperature were taken in all animals from day 0 Blood for isolations was obtained by cardiac puncture on three of days +2 to +7 On one of days +6 or +7 the rabbits were killed by the intravenous injection of 2 5 ml of a 6 per cent solution of mebumal (sodium salt) Immediately before doing this blood was obtained by cardiac puncture and used for a third isolation experiment and for serological examination The following material was taken from the killed animals for morphological examination lymph (also for isolation the popliteal and superior iliac lymph nodes and the spleen Bone marrow smears were taken from four rabbits of group 1

It is known that the administration of cortico-steroids can inhibit delayed type hypersensitivity reactions by decreasing the local cellular infiltration and so the toxoplasmin test was not repeated during the trial period

RESULTS

During the course of cortisone treatment certain changes in the white blood cell picture were demonstrated Table 1 shows a comparison between mean numbers for the different white blood cell types before and after 5 days cortisone treatment A highly significant decrease in lymphocytes was demonstrated together with a significant increase in pseudo eosinophilic leukocytes both rod and segment nucleated forms Immature forms of granulocytes were not seen Fig 1 records the

1 DT — Dye test
2 CFT — Complement fixation test

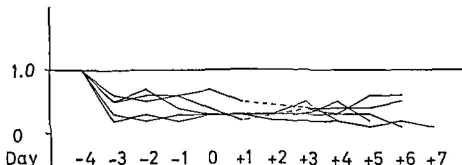


Fig 1

Lymphocyte counts on day −4 in each case taken as a unit (horizontal line) Individual curves on following days recorded as fractions of this unit

lymphocyte level for each day in the rabbits of group I It will be seen that the decrease in lymphocytes *already began on the 2nd and 3rd day* of cortisone treatment and that the level was then maintained These findings correspond well with observations made by several investigators (4, 15, 27) —An expected decrease in eosinophils is indicated, but it is not significant (Table 1)

TABLE 1

Distribution of Different White Blood Cell Types on Days −4 and 0

Day	Rod sh	Segment sh	Eosinoph	Basoph	Lymphoc	Monoc
4	98±22	3 127±589	25±13	147±38	8 800±359	205±40
0	452±116	6 860±1,323	9±5	221±73	2 740±511	201±46
t	2 993	2 845	1 103	0 895	9 824	1,392
P	*0 01*	*0 01*	*0 3*	*0 4*	*0 001*	*0 2*

Clinical course The rabbits of groups I and II (seronegative and seropositive after immunization with killed parasites respectively) revealed fatigue and malaise from day 5 or 6 after inoculation One rabbit of group II died spontaneously on day 7 However, despite the fact that these animals were severely ill, local symptoms in the mucous membranes or nervous system, common in untreated toxoplasmic rabbits, were not seen Fever reactions ($\geq 40°$ C) were recorded at the earliest on day 5 and were only found in two animals of group I and in three animals of group II

The remaining rabbits, belonging to group III (high antibody titres after previously sustained infection) all seemed to be in good health during the whole trial period *No signs of illness were seen Daily temperature controls* did not reveal any fever reactions

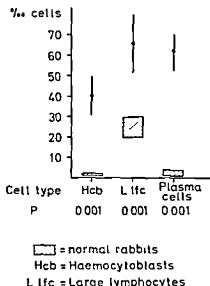


Fig 2

Means and standard errors of haemocytoblast large lymphocyte and plasma cell ratios in imprints from popliteal lymph nodes in Group III The shaded area covers the mean and standard error of a normal material

Lymph nodes Enlargement of lymph nodes was demonstrated in all rabbits, although this was less pronounced than in toxoplasmic rabbits that had not received cortisone In animals of group I, the popliteal lymph nodes were enlarged by about ⅓ of their normal size The consistency was somewhat firm Necroses were seen, but were less visible than in untreated rabbits, probably due to less oedema — Rabbits of group II revealed enlargement of the popliteal lymph nodes to about double the normal size, with considerably firmer consistency than normal Necroses seemed to be larger and more numerous than in group I, but less visible than in untreated immunized rabbits — In the animals of group III expected to have a good basic immunity, only a slight enlargement of the popliteal lymph node, without change in consistency and without necroses, was observed — The superior iliac lymph node only showed macroscopical changes in group II, in all the animals of which the node was somewhat enlarged, with visible small necroses

Imprints from lymph nodes showed scarcity in lymphocytes compared to normal nodes In the nodes of groups I and II, the lymphatic tissue was also severely damaged by invading parasites The preparations were poor in cells and considered not suitable for differential cell counts, which were only carried out on imprints from the rabbits of group III in which parasitic invasion was slight It will be seen from Fig 2 that the percentages of haemocytoblasts, large lymphocytes and plasma cells in the regional lymph node were significantly higher than for normal animals ($P < 0.001$ for all three cell types)

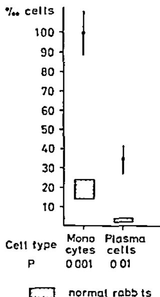


Fig 3

Means and standard errors of monocyte and plasma cell ratios in spleen imprints from Group III The shaded area covers the mean and standard error of a normal material

Toxoplasma organisms were seen in abundance in both regional and superior iliac lymph nodes from groups I and II In many preparations from group II both parasite multiplication and damage to the lymphatic tissue were so pronounced that more parasites than host cells were seen The majority of parasites were seen extracellularly although intra cellular localization was also common The host cells macrophages haemocytoblasts and large lymphocytes regularly contained larger numbers of parasites than were seen in corresponding cells from rabbits not treated with cortisone Thus 100 and more parasites were seen in one single macrophage and up to 50 toxoplasms in one haemocytoblast Moreover both small lymphocytes and pseudo eosinophilic leukocytes were commonly found to contain single parasites —In imprints from group III parasites were only seen in preparations from the regional lymph node of three rabbits 177 178 180) The toxoplasms were few and always located near the marginal sinus Intracellular localization in large lymphocytes was found in all three animals

Spleen Appreciable enlargement was demonstrated in group II alone in which the spleens of all rabbits had increased by about half the normal size Macroscopic necroses also were restricted to this group alone but they were found in smaller numbers than in rabbits not treated with cortisone

Despite the fact that macroscopical findings were less pronounced in the cortisone treated rabbits imprints from groups I and II revealed

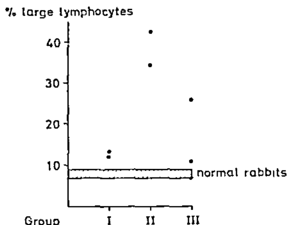


Fig 4

Percentage of large lympocytes in thoracic duct lymph in the three experimental groups The shaded area covers the mean and standard error of a normal material

severe tissue damage by an abundance of invading parasites For this reason, imprints from these two groups could not be used for differential cell counts Fig 3 shows that the relative numbers of monocytes and plasma cells in group III were significanttly higher than for normal rabbits ($P < 0\ 001$ for monocytes and 0 01 for plasma cells) More parasites than host cells were seen in several imprints from group II Many host cells were crowded with parasites—*Toxoplasma* was not seen in imprints from group III

White blood cell picture The white blood cell changes caused by cortisone treatment have been described above During the trial period after the inoculation with *Toxoplasma*, no significant change in the white blood cell picture was demonstrated Nor was there monocytosis or any sign of increased phagocytic activity by monocytes (For the lymphocyte level in group I, see Fig 1) Basophilic "toxic" granules appeared in pseudo eosinophilic leukocytes in three rabbits of group I (22 per cent 60 per cent of all pseudo eosinophilic leukocytes on days +5 to +7) and in four rabbits of group II (40 per cent–60 per cent of the same cell type and on the same days) Toxic granules were not seen in smears from group III

In one blood smear from day +7 (rabbit 80, group I) *Toxoplasma* was seen intracellularly within a pseudo eosinophilic leukocyte

Bone marrow[3] The ratio for myelo + metamyelocytes in the pseudo-eosinophilic series was higher than for normal uninfected rabbits ($P = 0\ 05$) No other change in the bone marrow cell picture was demonstrated It should nevertheless be borne in mind that the test group was very small (4 rabbits)

3 Differential cell counts performed by Dr *G Winqvist* Royal Veterinary College Stockholm



Fig 5
Toxoplasma infected large lymphoid cell from thoracic duct lymph



Fig
Macrophage with multiplying Toxoplasma organisms from thoracic duct lymph The number of multiplying Toxoplasma organisms in this and the preceding Fig is considerably larger than in corresponding cells from rabbits not treated with cortisone

Toxoplasma organisms were seen in bone marrow smears in considerably greater numbers than in experimentally infected rabbits not treated with cortisone Most parasites were found intracellularly and were seen both in reticulum cells and in various types of white blood cells However while parasite multiplication was often seen in other

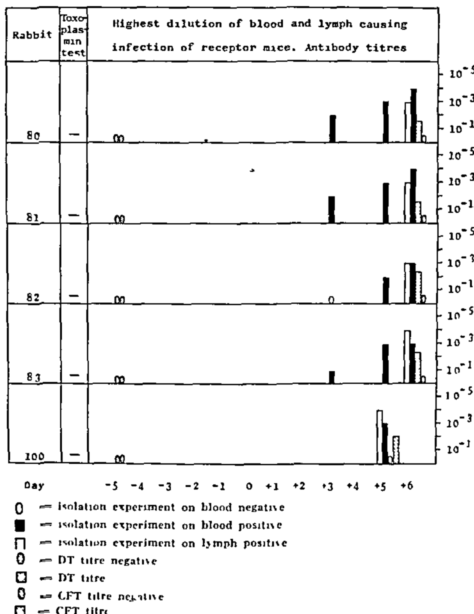
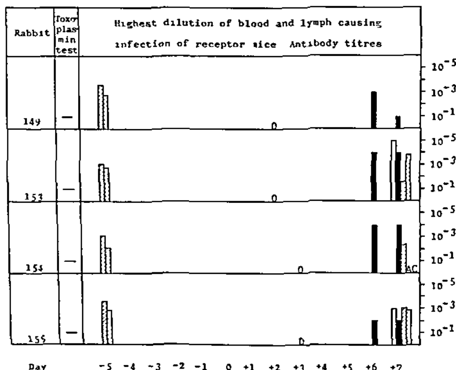


Fig 7

Toxoplasmin tests, isolation experiments and antibody titres in the animals of Group II

cell types, this was only rarely demonstrated in cells belonging to the pseudo-eosinophilic cell series. Within these cells, the parasites stained poorly and were not clearly defined in the surrounding cytoplasm in which the granules appeared unchanged. It seems as if the parasites had been attacked and partially destroyed by the host cells.

Thoracic duct lymph. Only 7 lymph smears were technically good



0 — isolation experiment on blood negative

█ — isolation experiment on blood positive

⊓ — isolation experiment on lymph positive

▨ — DT titre

▧ — CFT titre

AC — serum anticomplementary

Fig 8

Toxoplasmin tests isolation experiments and antibody titres in the animals of Group III

enough to allow differential cell counts Fig 4 indicates the relative numbers of large lymphocytes found in these specimens A tendency to higher levels than in uninfected controls is seen The material is too small to allow further conclusions

Toxoplasma organisms were seen in one of three examined lymph smears from group I and in two of two examined specimens from group II The parasites were chiefly found intracellularly in large lymphocytes but occasionally also in macrophages (Figs [illegible] and 6) The occurrence of *Toxoplasma* in smears from group II by far exceeded that in the other groups

Isolations Figs [illegible] 6 and [illegible] present the results of isolation experiments from blood and lymph in the three experimental groups As will be seen there is a pronounced difference between on the one hand groups

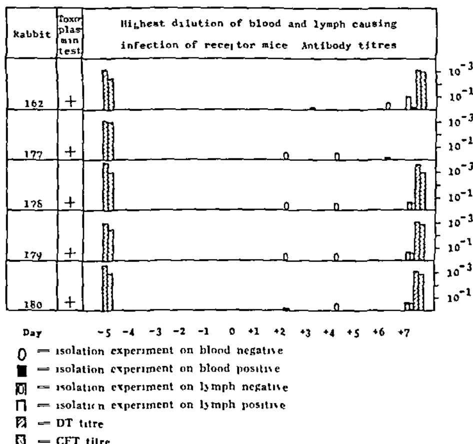


Fig 9

Toxoplasmin tests, isolation experiments and antibody titres in the animals of Group IV

I and II in which *Toxoplasma* was isolated from lymph and blood in high dilutions in all animals indicating a violent infection, and, on the other hand group III in which the parasite was isolated from blood or lymph in low titre and only in three of five rabbits examined.—The level of parasitaemia was higher in the cortisone-treated animals of groups I and II than in the corresponding groups described previously, in which the rabbits were not treated with cortisone (14). However, no difference was found between untreated animals and the animals of group III.

Toxoplasmin reactions and antibody titres. Immediately before the onset of the experiment, the toxoplasmin test was negative throughout for groups I and II and positive in all animals of group III, as will be seen from Figs. 5, 6 and 7.

The antibody titres before and on the last day of the trial period also appear from these figures. There was a moderate increase in DT titres in four of five rabbits in group I, whereas in the 5th rabbit the

DT remained negative on day +7 On this day the CFT was negative in the four DT-positive rabbits, but positive in high titre (1 120) in the DT-negative animal That such an unexpectedly early appearance of a positive CFT sometimes occurs has been shown in a previous investigation (12).

Group II showed a tendency towards decrease in DT titres, but the CFT remained unchanged

In group II, most titres remained unchanged during the trial period A 2-fold rise in the CFT was found in one animal

DISCUSSION

In the preceeding and this publication a series of experiments is reported on immunity against *Toxoplasma* (RH strain) in rabbits seropositive 1) after natural infection and 2) after immunization with killed *Toxoplasma* organisms Seronegative rabbits were also studied for comparison In the second paper the animals were treated with cortisone both before and during the period of infection For simplicity, the various trial groups were given the same numbers in the two papers

From the clinical picture and the results of isolations and imprints it was found that animals with high antibody titres after a passed infection (Group III) also exhibited good resistance towards challenge On the other hand animals with the same antibody level after immunization with killed *Toxoplasma* organisms (Group II) showed as great a susceptibility towards infection upon challenge as the seronegative animals With the exception of one animal (No 161), this susceptibility was also found to be true for rabbits with low to moderate titres after infection However, the possibility exists that a smaller challenge dose might have disclosed some resistance in one or both of groups II and IV

These results suggest that the presence of living parasites was of vital importance for the development of protective immunity in the naturally infected rabbits Little is known about the antigenic difference between living and killed *Toxoplasma* The method of killing the organisms may destroy one or more antigens among other things, by denaturation and this factor must be taken into consideration However, other differences also probably arise Recent studies in the field of parasite immunology indicate that secretion excretion products from the parasitic organism might play a prominent part in immunity (31, 35) The more complete antigenic spectrum characterizing the living infective agent might well give rise to a broader antibody response as well as a more effective cell bound immunity

The fact that no significant difference in antibody level between groups II and III could be demonstrated indicates that circulating anti bodies did not play a decisive part in the immune protection The dye test records the cytotoxic effect of antibodies in the presence of accessory factor This test is believed to be a measure of the concentration of

neutralizing antibodies In spite of the presence of such antibodies multiplication can occur because the intracellular parasite is protected against antibodies and the passage between cells can take place faster than the neutralizing effect of antigen-antibody-assessory factor reaction However, the part played by the circulating antibodies in protective immunity must be left open for the present

A numbers of factors indicate that cell-bound immunity may play an important part in protective immunity The toxoplasmin tests showed (series 2) that animals with high antibody titres after an infection had developed delayed type hypersensitivity, while immunized rabbits were toxoplasmin negative The *Toxoplasma* organisms inoculated were found to be transported fairly rapidly to the nearest draining lymph node as shown previously (13), where they were attacked by sensitized lymphocytes It is known that up to a 48 hours' contact between target cell and lymphocytes is necessary for a cytotoxic effect to be demonstrated (22) This should allow sufficient time for some intracellular multiplication and several parasite passages between cells Of interest in this connection is the observation made by several workers (35) recently confirmed by *Pulvertaft* (1962) and in electron microscopic studies by *Marchesi & Gowans* (1964) that lymphocytes are able to force their way into cells It may also be of importance that complement is not necessary for this type of cytotoxic reaction

Protective immunity in several parasitic diseases is considered to be due to the continued occurrence of living infectious material in the tissues The phenomenon is referred to as premunition in parasitological studies (*E Sergent et al* 1924, 1956, *E Sergent* 1963) and has been seen as a state of balance between the parasite and the defence mechanisms of the host which permits already established parasites to live but under such restricted conditions that the infection cannot readily spread Should the parasite die the host loses its resistance and becomes susceptible to fresh attack by the same infective agent —It has been shown (30) that latent protozoan infections with consequent premunition can last for years —The nature of premunition is not clear, but many workers consider it to be a true immunological phenomenon dependent on the relatively weak antigenic properties of the infective agent

The possibility exists that there may be an intracellular metabolic mechanism of interferon-like nature that is induced by the infecting agent, but no data supporting this hypothesis have been published Another possible mechanism may involve substances like ablastin, an antimetabolite described by *Taliaferro* in 1928 *Moulder* (1948) demonstrated that the presence of ablastin changed the metabolism of glucose from that characteristic of reproducing trypanosomes to that characteristic of an adult population Although the parasites were not able to reproduce their vitality was not greatly affected In his investigations on ablastin (32, 33) *Taliaferro* refers to this substance as an antibody,

in spite of the fact that it differs in several respects from the classic antibody nature

The good resistance of naturally infected rabbits with high antibody titres may possibly be due to premunition It has been suggested by *Beattie* (1963) and later by *Piekarski* (1966) that *Toxoplasma* may bring about this type of phenomenon This suggestion is supported by the fact that living *Toxoplasma* organisms have been demonstrated in the tissues of animals (*Lainson* 1959) and man (*Hogan* 1958 *Remington et al* 1960 1964) many years after an infection The observations of *Cutchings & Warren* (1956) and *Wilfuhr* (1957), on the other hand indicate that some protective immunity can be achieved with killed *Toxoplasma* Their results are however somewhat controversial

With the exception of one animal (No 161) it was not possible to demonstrate protective immunity in rabbits with low to moderate antibody titres after natural infection Although these animals have lower antibody titres than those in Group III it is conceivable either that they may have had a weak immunity which was overcome by the challenge dose employed or perhaps the necessary requirements for premunition were not present although residual antibody titres remained

As in a number of other parasitic diseases marked reactions in reticulo endothelial and lymphatic tissue occur at an early stage in the course of toxoplasmosis These were reflected in increased phagocytosis proliferation particularly of the monocytes and changes within the lymphocyte series Several of these changes were demonstrated also in the very modified toxoplasmosis that occurred in rabbits with good resistance to challenge This observation indicates that small amounts of reproducing toxoplasms were sufficient to stimulate cells of the reticulo endothelial and lymphatic system Of particular interest is the significant increase of immature lymphocytes in the lymph nodes It is possible that the changes are partly due to transformation of small lymphocytes by antigenic stimulation in the manner described for transplantations reactions (9)

In groups II and III in which the animals had high antibody titres before challenge there was a significant increase in plasma cells over that in normal rabbits seen in imprints from lymph nodes and spleen This phenomenon which accompanies the accelerated antibody production during the secondary response is well known and has been studied by *Fagraeus* (1948) and others

Another expression of antibody producing cell stimulation is the significant increase of the haemocytoblast in imprints from lymph nodes in all experimental groups

In animals that developed full infection in spite of the presence of humoral antibodies it was possible to demonstrate necroses in lymph nodes and spleen to a far greater extent than in animals that were seronegative before the trial The intracellular multiplication and rupture of host cells always leads to necroses if the infection is pronounced In the

animals of this study that had humoral antibodies before challenge the tissue destruction probably was occasioned also by local antigen-antibody reactions

Three experimental groups were treated with cortisone The steroid effect was observed first as a peripheral lymphocytopenia as early as 24 hours after the beginning of treatment After the animals had been infected with *Toxoplasma* further signs appeared Both fever reactions and local symptoms were less pronounced than in animals not given cortisone The same was true of the enlargement of lymph nodes and spleen, probably resulting from lymphocytolysis as well as less oedema because of reduced capillary permeability —No further reduction in the blood lymphocytes was observed during the period of illness in spite of the fact that toxoplasmosis can give rise to lymphocytopenia (2) There was, on the other hand, a significant increase in the number of immature lymphoid cells in the lymph nodes, which probably was due both to the effect of cortisone and antigen stimulation by the toxoplasms

No definite conclusion can be drawn about the effect of cortisone on the formation of circulating antibodies from the results of this study The antibody titres of Group I (animals seronegative before the trial) on the final day of the trial were similar to those animals not treated with cortisone This result may be explained by the greater amount of antigen supplied to the antibody-producing tissues of the former group caused by the vigorous parasite multiplication —A secondary response was to be expected in the other two groups In comparison with seronegative animals, a significantly greater number of plasma cells was found in the regional lymph nodes and spleen This increase might be an indication that such a reaction normally takes place up to a certain stage in the development Titre increase as an expression of increased antibody production was lacking in nearly all cases treated with cortisone however This observation is in accordance with results of other workers (10) However, it should be pointed out that towards the end of the trial period increase in titres occurred only in about half of the animals not treated with cortisone in corresponding trial groups

This investigation has not given clear support to the hypothesis that cortisone greatly influences cell bound immunity In the first two trial groups the toxoplasmin reaction was negative in all animals, which indicates that they lacked cell bound immunity Of course, it cannot be excluded that the development of this kind of immunity was postponed by the administration of cortisone and that this delay in turn, favoured the vigorous parasite multiplication that characterized the infection in these groups However, there was reason to believe that the main cause of the accelerated parasite multiplication was the influence of cortisone on certain intracellular phenomena All the animals of Group III had a positive toxoplasmin reaction, indicating cell-bound immunity It seems plausible to assume that this immunity should have been in-

fluenced by the reduction of small lymphocytes that accompanies the administration of cortisone and yet all animals in this group exhibited a marked resistance to challenge which in no way could be shown to be less than in untreated animals with high antibody titres after natural infection.

It should be reasonably clear that the rapid parasite multiplication in Groups I and II can not be ascribed unequivocally to the effect of cortisone on the development of immunity.—In imprints from lymph nodes and spleen it appears quite clearly that infected cells, chiefly the macrophages, haemacytoblasts and large lymphocytes, contain considerably more parasites than the corresponding cells of animals not treated with cortisone. Increased phagocytosis cannot be the explanation for this difference since the investigation showed that cortisone had an inhibiting effect on proliferation as well as phagocytosis in the monocytes and probably in the macrophages also. Other host cells have little phagocytic capacity. Thus the large parasite content in the host cells *probably reflects the result of increased intracellular multiplication.* The exact mechanism of the intracellular action of cortico steroids is not fully understood. It has been shown, however, that they depress the ability of the cell to break down ingested micro-organisms (*Furie*). What is probably of cardinal importance is the ability of cortisone to increase the stability of the lysosomes (*Thomas*). It is noteworthy that the enhanced parasitic growth after cortisone treatment was not apparent in animals with high antibody titres after infection (Group III). It is true that there was some multiplication of parasites, at least in a few instances, but this growth was similar to that in the corresponding trial group of animals that were not treated with cortisone. The resistance in these trial groups was exceptionally effective and the possibility exists that non immunological mechanisms may play some role in the defence system. The question as to whether living *Toxoplasma* organisms can stimulate production of interferon type substances deserves further investigation.

SUMMARY

After pre-treatment with cortisone, three groups of rabbits were inoculated with *Toxoplasma*, namely:

1. Seronegative, toxoplasmin negative animals.
2. *Rabbits with high antibody titres, DT > 1:1250 (CFT ≥ 1:120)* after immunization with killed *Toxoplasma* organisms. These animals were also toxoplasmin negative.
3. Rabbits with high antibody titres, DT ≥ 1:1250, CFT ≥ 1:120 and positive toxoplasmin reaction after a passed natural infection.

The cortisone treatment was continued during the whole trial period. This treatment was observed to cause lymphocytopenia and depression of certain cellular defence reactions typical of toxoplasmosis.

Cortisone appeared to enhance the infection in seronegative and immunized rabbits, whereas in rabbits seropositive after a passed infection, the resistance to challenge was equally as effective in cortisone treated and untreated rabbits Therefore the difference between animals immunized with killed *Toxoplasma* and active infection was made more obvious by cortisone treatment Possible explanations are discussed

The present experiments suggest that circulating antibodies do not play an important role in immune protection

The toxoplasmin test demonstrates a delayed type hypersensitivity in rabbits with high antibody titres after infection, but not in immunized rabbits This difference favours the supposition that cell associated immunity was important in immune protection

The possibility that premunition is an important mechanism in resistance to *Toxoplasma* infection is discussed together with the suggestion of a protective mechanism of non-immunological nature

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BRIEF REPORT

VENOUS OUTPUT OF ^{3}H-THYMIDINE-LABELLED LYMPHOCYTES FROM THE THYMUS

By *Bengt Larsson*

In previous studies (1, 2) an arterio-venous difference in the number of lymphocytes was demonstrated in the thymic vessels, with a higher percentage and larger number of small lymphocytes in the blood leaving the thymus. The blood cells were stained supravitally. A minimum output from the thymus of 73 cells/mm^3 of blood was calculated (3). As the blood flow through the thymus was found to be 82 mm^3/minute, this represents a release of 6000 lymphocytes/minute (4).

An autoradiographic method was described by *Cannon & Wissler* (1965) for studying the output of cells from the spleen after irradiation of all other lymphocyte-producing organs (5). The simple comparison between afferent and efferent blood vessels was not, however, used for this purpose. A report is given in the present paper of an autoradiographic technique for comparison between the content of labelled cells in afferent and efferent thymic vessels, after a single injection of tritiated thymidine.

A total of 40 male guinea pigs (weight 210–250 g) were used. All the animals received an intraperitoneal injection of tritium-labelled thymidine (TRA 120 Thymidine (methyl-T), The Radiochemical Centre, Amersham, England) in a dose of 0.5 mC/kg b.w. They were divided into four experimental groups and examined 24, 48, 72 and 96 hours after the isotope injection. The animals were anaesthetized with sodium Nembutal® (0.25–0.50 mg/100 g b.w. i.p.). The thymus was exposed and a thymic vein incised. Blood samples were collected in a heparinized blood pipette (Heparin ® 5000 IE/ml, Vitrum, Stockholm, Sweden) for preparation of blood smears and for white cell counts in a Bürker chamber. The right carotid artery was incised close to the origin of the thymic artery, and blood samples were taken for the same analysis as mentioned above. The artery was then ligated. Finally, the thoracic duct was exposed at its confluence with the left subclavian and jugular veins, mainly according to the technique of *Reinhardt & Yoffey* (6). The duct was incised and lymph collected for preparation of smears.

For counting of white blood cells in the Bürker chamber 25 mm^3 of blood was collected in a pipette and diluted with 475 mm^3 of Türk's solution containing methyl violet for staining of the white cells. Totally 288 squares were counted for each determination.

All the smears were allowed to dry in air, and were then fixed in methanol for 5 minutes. After drying, the smears were covered with a thin film of celloidin (0.5 per cent celloidin in an alcohol-ether solution) and dried again. The slides were dipped into the photographic emulsion (Ilford Nuclear Research Emulsion K2 diluted in two parts of re-destilled and filtered water, dark room) and dried for 3 hours at 20° C. Exposure proceeded for 1 month in absolute darkness at +4° C. The slides were developed (Kodak D 19) for 3 minutes, fixed for 15 minutes and washed in running water for at least 20 minutes. While still wet, the slides with the blood and lymph smears were stained in Giemsa solution (200 drops in 100 ml of re-destilled and filtered water) for 60 minutes. Finally, the smears were dipped into alcohol of 70, 90, 95 and 100 per cent, covered with celloidin and a cover slip put on.

The cell counts were performed in a light microscope at a magnification of about 1000 ×. The background labelling was as a rule scanty. A minimum load of 3 grains/cell was required for the cell to be regarded as labelled. Altogether 100 cells

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were counted in each smear The number of labelled and unlabelled lymphocytes monocytes and granulocytes was registered The absolute numbers of labelled lymphocytes were calculated from the total number of white cells and the percentage of labelled lymphocytes

The results were analysed statistically by Student's t test and were as follows (mean ± S E)

Source of blood	Days after ^{3}H thymidine treatment	No of animals	Blood: No of WBC	Blood: Lymphocytes %	Blood: Lymphocytes No/mm^3	Blood: Labelled lymphocytes %	Blood: Labelled lymphocytes No/mm^3	Lymph: Labelled lymphocytes %
Thymic vein	1	10	1995 ±175	87 1 ±1 9	1739 ±170	4 3 ±0 3	97 ±10	
Carotid artery			1570 ±199	72 1 ±2 1	1124 ±137	1 5 ±0 3	25 ±6	
Thymic vein	2	10	1713 ±240	89 1 ±1 7	1516 ±202	13 5 ±1 1	246 ±44	4 0 ±0 5
Carotid artery			1473 ±199	74 1 ±3 2	1092 ±140	6 5 ±0 9	90 ±15	
Thymic vein	3	10	1729 ±148	90 5 ±1 2	1569 ±140	18 0 ±1 0	310 ±29	8 2 ±0 6
Carotid artery			1413 ±216	70 2 ±2 8	1017 ±177	10 0 ±1 0	157 ±30	
Thymic vein	4	10	1601 ±201	87 1 ±2 2	1410 ±185	10 6 ±1 2	174 ±41	10 8 ±1 0
Carotid artery			1501 ±150	76 7 ±5 0	1109 ±101	7 3 ±1 0	109 ±18	

Thus both the percentage and the number of labelled lymphocytes in the thymic vein blood exceeded the corresponding values in the carotid artery blood 1 2 and 3 days after injection of tritiated thymidine ($p<0\ 01$ $p<0\ 001$ On the 4th day t there was a higher percentage and number of labelled lymphocytes in the thymic vein than in the carotid artery These differences were not however significant

The findings indicate that 1 day after isotope treatment there is an outflow of labelled lymphocytes from the thymus This outflow increases 2 and 3 days after injection and decreases on day 4 In a previous work (2) the majority of cells ... n t e small lymphocytes Thus ... formed in the thymus leaves the ... NA synthesis From the table the ... mus n days 1 2 and 3 after the is t p injection is calculated to be at least 34 26 and 36/mm^3 of blood respectively ... of he 99 ... e c nt r nf fence interval

Th ... increased from the 2n ... after the 3rd ... tes fell Thus ... he lymph exceeded the corresponding ... is may be interpreted as a passage of ... s uring the theory of recirculation f

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